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OXYGEN CONCENTRATION AND BIOLUMINESCENCE INTENSITY

II. CYPRIDINA HILGENDORFII

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TWO FIGURES

INTRODUCTION

It has generally been assumed that light emission in organisms is the result of a direct oxidation of a substrate molecule (luciferin) in the presence of an organic catalyst (luciferase). In most luminous [2] organisms where it has been studied it has been shown that light emission does in fact cease in the absence of oxygen. Very little previous study however has been concerned with the elucidation of the role of oxygen in bioluminescence. Quantitative information concerning the relationship between oxygen and luminescence would be expected to yield information valuable in gaining a better understanding of the reactions in bioluminescence.

In the intact organism the finding that light emission ceases in the absence of oxygen is necessarily only indirect evidence for the supposition that oxygen is concerned directly in the luminescent reactions. In the first paper of this series (Hastings, '52) a quantitative study of the oxygen-light intensity relationship in bacteria and fungi was reported. The results agreed very well with a formulation based upon the assumption that oxygen as well as luciferin is a substrate, and that

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[2] Very little is known about certain exceptional cases where extracts will luminesce in the absence of oxygen (see Harvey, '26; Harvey and Korr, '38). For a general treatment of bioluminescence, see Harvey ('40, '52).

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the reaction proceeds only when both are combined at specific sites on the luciferase molecule. A schematic representation of the reactions was written

$$\mathrm{LH_2} + \mathrm{O_2} \underset{\text{(luciferase, A)}}{\rightleftharpoons} \mathrm{LH_2 \cdot A \cdot O_2} \qquad (1)$$

$$\mathrm{LH_2 \cdot A \cdot O_2} \rightarrow \mathrm{C^* + P + A} \qquad (2)$$

$$\mathrm{C^* \rightarrow C + h\nu} \qquad (3)$$

where LH_2 represents the luciferin, A the luciferase, O_2 the oxygen, C^* the excited molecule resulting from the breakdown of the complex, and P the other products of the breakdown. On the basis of the above assumption, with the additional provision that a competitive type of combination or adsorption of the substrates occurred, the expected relationship between light intensity, I, and oxygen concentration, $[O_2]$, was given (Hastings, '52) as

$$I = \frac{k_3 K_1 K_2 [A]\,[LH_2]\,[O_2]}{(1 + K_1[O_2] + K'_1[LH_2])\,(1 + K_2[LH_2] + K'_2[O_2])} \qquad (1)$$

where $[LH_2]$ and [A] are the concentrations of luciferin and luciferase respectively, k_3 a rate constant, and K_1, K_2, K'_1, and K'_2 equilibrium constants.

With constant luciferin concentration and on the assumption that competitive adsorption is appreciable only at high concentrations, the equation reduces to the form

$$I = \frac{I_{max}\,[O_2]}{K_s + [O_2]} \qquad (2)$$

which is the usual form of the Michaelis-Menten relation, where I_{max} represents the maximum velocity of the reaction and K_s a dissociation constant. Lineweaver and Burk ('34) have shown that a more useful form of the equation results if its reciprocal is multiplied by $[O_2]$, so that

$$\frac{[O_2]}{I} = \frac{[O_2]}{I_{max}} + \frac{K_s}{I_{max}} \qquad (3)$$

When the equation is satisfied, a plot of $[O_2]/I$ against $[O_2]$ results in a straight line. The maximum velocity of the reaction, I_{max}, is given by the reciprocal of the slope of the resulting

line, and from the intercept, equal to K_s/I_{max}, the value of K_s may be found.

The present paper reports the results of experiments similar to those reported for the bacteria and fungi, using as experimental material partially purified extracts from the luminous ostracod crustacean, *Cypridina hilgendorfii*. While the luciferin used was a relatively crude extract as compared with that available from Anderson's ('35) purification procedure, many complicating factors which exist in using the intact cell are excluded here, and we may be reasonably certain that we are dealing more directly with the luminescent reactions.[3] Chase ('49a) has given evidence for the formation of a luciferin-luciferase complex by showing that the light intensity as a function of luciferin concentration is described by a relation having the form of equation (2). The present paper demonstrates that oxygen must also be considered to be associated in this complex.

MATERIALS AND METHODS

Luciferin is very unstable in the presence of oxygen and undergoes an auto-oxidation without light emission. It was thus necessary to take every precaution in handling the material to minimize this oxidation. The luciferin extract was prepared [4] by benzene and methanol extraction of dry *Cypridinae* which yielded a highly deliquescent powder of a brownish-red color. The stock luciferin solution for use in these experiments was prepared by dissolving 25.6 mg of the powder in 40 ml of 0.1 N HCl. This solution was immediately saturated with purified hydrogen and placed under an atmosphere of pure hydrogen. For a series of experiments a quantity was removed and placed in a test tube immersed in an ice bath. The estimation of the concentration of active luciferin was made according to Anderson's ('35) method, by measuring the

[3] For a comprehensive review of chemical and kinetic studies upon the *Cypridina* system, see Chase ('48).

[4] I am indebted to Dr. Howard S. Mason for providing me with the luciferin extract.

total light emitted per unit weight of luciferin. The dry *Cypridina* organisms from which the extract was made yielded 250 mv total light per milligram, while the stock solution made up from the extract gave 1250 mv per milligram. At the end of the experiments a second measurement of activity agreed within 1%, indicating that no appreciable quantity of luciferin was lost through auto-oxidation.

The luciferase stock solution was prepared by two successive extractions of 5 gm of the dried powdered organisms in a total of 100 ml of distilled water, followed by prolonged dialysis against tap water, phosphate buffer, and distilled water. Dilutions of the stock solution were used in the experiments.

As the light emission is known to be greatly affected by the ionic composition, including the pH (Anderson, '33, '37; Chase, '48a), the reaction mixture used in these experiments corresponded to that used by Chase ('49a), consisting of phosphate buffer at pH 6.8 (equal parts M/15 KH_2PO_4 and M/15 Na_2HPO_4) with 0.01 M NaCl added.

The occurrence of auto-oxidation during the course of an experiment has been shown (Anderson, '36; Chase, '49) to result in a complicated type of light emission. The experimental procedure was therefore designed so that the quantity of non-enzymatically oxidized luciferin present could be considered as negligible. Twenty-five milliliters of the reaction mixture were placed in the tube and equilibrated with the desired gas mixture by bubbling through a sintered glass disc for 4 minutes, at which time 1 ml of the stock luciferin solution was added. Thirty seconds later 1 ml of the luciferase solution was added, starting the luminescent reaction. Readings of light intensity were made every 15 seconds, and 1¼ minutes after the start of the reaction the gas mixture was replaced by air. Readings of light intensity were then continued for another minute or two. The tube was then disconnected, cleaned, and prepared for another experiment with a different oxygen concentration. The experiments were run at room temperature, approximately 24°C.

The general techniques for measuring light intensity and for controlling oxygen concentrations were the same as described in detail previously (Hastings, '52). Light intensity was measured with a photomultiplier tube (RCA 931-A) and a metering circuit. Gas mixtures containing known concentrations of oxygen were prepared by mixing purified hydrogen or oxygen with air, or at low oxygen concentrations by mixing hydrogen with electrolytically produced oxygen.

RESULTS AND DISCUSSION

Figure 1 shows three examples of data taken in the manner described above. At the top is a control run in air. While the decay of intensity is theoretically logarithmic, the luciferase concentration was sufficiently low so that an apparent straight line resulted. To determine the reaction velocity at the different oxygen concentrations, the intensities in air and in the second oxygen concentration were compared by extrapolation to the same point on the time axis. It can be noted that the slopes of the decay curves differ in a way which indicates that the rate of utilization of luciferin increases with higher oxygen concentration.

The raw data taken in this way (for two series of experiments with a 1/20,000 luciferase dilution) are calculated by means of equation (3) and plotted in figure 2 B. It can be seen that the data are well described by the equation. The value of K_s from this plot is 2.25×10^{-5} moles/liter at 24°C. The slope of the line is 0.91, indicating that the maximum intensity to be expected for the reaction is 10% above the intensity found in air. The intensity in 100% oxygen was found to correspond exactly to this value. In figure 2 A the above data have been recalculated and plotted, using the calculated figure as the value for 100% intensity.

A third run several weeks later differed slightly in the value of K_s in the equation, but was of the same quality as the two runs plotted in figure 2. The difference was attributed to an increase in room temperature, which was higher by 3–4°C. Experiments using luciferase dilutions of 1/4000 and 1/10,000

gave no difference in the value of the constant, and were of the same quality as those shown.

While the data can be seen to be in accord with the assumptions of the Michaelis-Menten relation, there is adequate reason

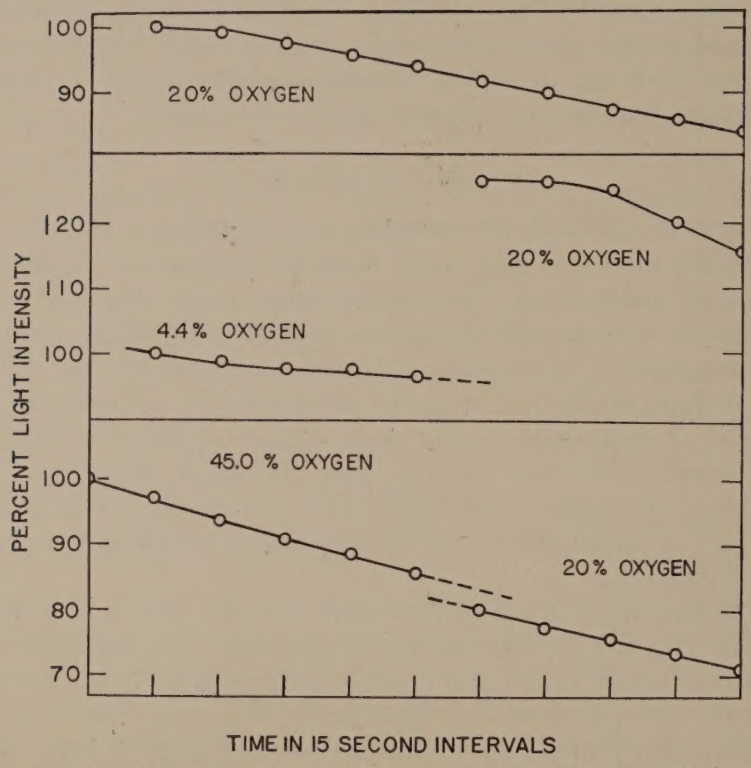

Fig. 1 Plot of light intensity against time when *Cypridina* luciferin is mixed with a very dilute luciferase solution. One hundred per cent intensity is taken as the first reading made 15 seconds after mixing.

Top: Control run in 20% oxygen.

Middle: Luciferin and luciferase mixed in 4.4% oxygen. Twenty per cent oxygen added 1¼ minutes later.

Bottom: Luciferin and luciferase mixed in 45% oxygen; 20% oxygen added 1½ minutes later.

to believe that the situation is somewhat more complicated. First of all any derivation for the kinetics of the reaction must account for both the luciferin and the oxygen. Secondly, although the data presented here bring no evidence to bear on

the point, it seems likely that competitive adsorption of the substrates may occur. Chase (personal communication) has observed that extremely high luciferin concentrations tend to

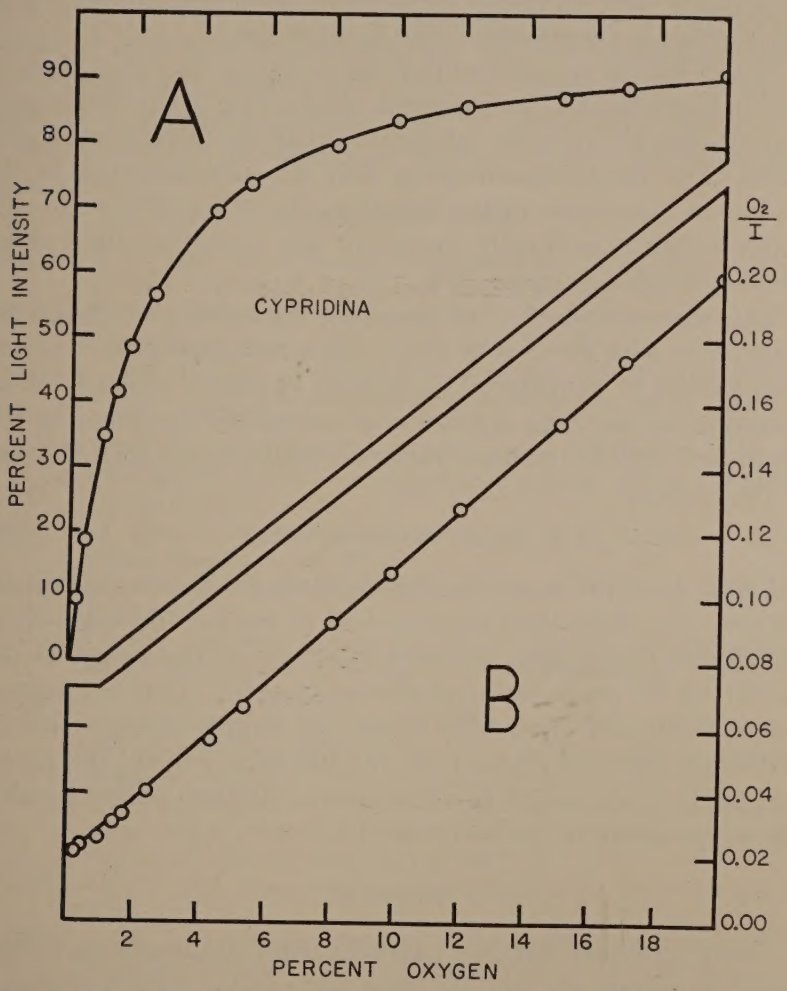

Fig. 2 Plot of data from a series of runs such as shown in figure 1.

A. Plot of per cent light intensity against per cent oxygen. One hundred per cent light intensity is the value of I_{max} calculated from the slope in figure 2 B.

B. Plot of the data according to equation (3) of text. O_2 is expressed in per cent oxygen and I in per cent light intensity in 20% oxygen. The data are well fitted by a straight line from the slope of which the maximum intensity is calculated.

decrease the reaction velocity from the maximum in the *Cypridina* system. Thus a relation having the general form of equation (1), either with or without the terms for competitive adsorption, would appear to be the most adequate for describing the kinetics in *Cypridina* at the present time.

It is of course recognized that the constant K_s may not represent a true equilibrium constant, depending upon the relative magnitudes of the rate constants involved (Briggs and Haldane, '25). On the assumption that K_s does measure, in this system, a true dissociation constant, its value falls within the range which is generally found for the oxidative class of enzyme systems (Haldane, '30).

The correspondence between the bacteria and fungi and *Cypridina* with respect to the oxygen-light intensity relationship should be considered as further evidence in favor of the assumption that the luminescent reactions in bacteria and fungi are similar to those outlined, with oxygen as a reactant in the system.

ACKNOWLEDGMENTS

I wish to express my indebtedness to Professor E. Newton Harvey who suggested the problem to me for his supervision and advice throughout the various phases of the study, as well as during the preparation of the manuscript. I am very grateful to Professor Aurin M. Chase for supplying me with the luciferase solution, as well as for his very helpful discussion and advice concerning various aspects of the problem, and in the preparation of the manuscript.

SUMMARY

Using partially purified extracts from the luminous crustacean *Cypridina*, a study has been made of the relation between light intensity (which is a measure of the reaction velocity) and the concentration of oxygen in solution. The relationship has the form of a rectangular hyperbola, where 50% of maximum intensity is reached at 1.9% oxygen. Such a relationship is in agreement with the assumption that the oxygen associates

in a complex with the enzyme before breaking down into the products of the reaction. As similar evidence has been given (Chase, '49a) for the formation of a complex between the luciferin and the enzyme, it is proposed that the kinetic expression should be based on the assumption that the enzyme contains sites specific for oxygen and luciferin respectively. Such an expression might also include terms for competitive adsorption of the substrates, as there is reason to believe that such may occur.

LITERATURE CITED

ANDERSON, R. S. 1933 The chemistry of bioluminescence. I. Quantitative determination of luciferin. J. Cell. and Comp. Physiol., *3:* 45.

———— 1935 Studies in bioluminescence. II. The partial purification of *Cypridina* luciferin. J. Gen. Physiol., *19:* 301–305.

———— 1936 Chemical studies on bioluminescence. III. The reversible reaction of *Cypridina* luciferin with oxidizing agents and its relation to the luminescent reaction. J. Cell. and Comp. Physiol., *8:* 261–276.

———— 1937 Chemical studies on bioluminescence. IV. Salt effects on the total light emitted by a chemiluminescent reaction. J. Am. Chem. Soc., *59:* 2115–2117.

BRIGGS, G. E., AND J. B. S. HALDANE 1925 A note on the kinetics of enzyme action. Biochem. J., *19:* 338–339.

CHASE, A. M. 1948 The chemistry of *Cypridina* luciferin. Ann. N. Y. Acad. Sci., *49:* 353–375.

———— 1948a Effects of hydrogen ion concentration and of buffer systems on the luminescence of the *Cypridina* luciferin-luciferase reaction. J. Cell. and Comp. Physiol., *31:* 175–192.

———— 1949 Studies on cell enzyme systems. I. The effect of ferricyanide on the reaction of *Cypridina* luciferin and luciferase and the combining weight of luciferin. J. Cell. and Comp. Physiol., *33:* 113–122.

———— 1949a Studies on cell enzyme systems. II. Evidence for enzyme-substrate complex formation in the reaction of *Cypridina* luciferin and luciferase. Arch. Biochem., *23:* 385–393.

HALDANE, J. B. S. 1930 Enzymes. Longmans, Green and Co., New York, pp. 35–37.

HASTINGS, J. W. 1952 Oxygen concentration and bioluminescence intensity. I. Bacteria and fungi. J. Cell. and Comp. Physiol., *39:* 1–30.

HARVEY, E. N. 1926 Oxygen and luminescence, with a description of methods for removing oxygen from cells and fluids. Biol. Bull., *51:* 89–97.

———— 1940 Living Light. Princeton University Press, Princeton, N. J.

———— 1952 Bioluminescence. Academic Press, Inc., New York.

HARVEY, E. N., AND I. M. KORR 1938 Luminescence in the absence of oxygen in the Ctenophore, *Mnemiopsis leidyi.* J. Cell. and Comp. Physiol., *12:* 319–323.

LINEWEAVER, H., AND D. BURK 1934 The determination of enzyme dissociation constants. J. Am. Chem. Soc., *56:* 658.

ELECTRICAL CONTROL OF MORPHOGENESIS IN REGENERATING DUGESIA TIGRINA

II. POTENTIAL GRADIENT VS. CURRENT DENSITY AS CONTROL FACTORS

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INTRODUCTION

The preceding paper (Marsh and Beams, '52) demonstrated that the polarity of regenerating *Dugesia tigrina* could be completely controlled by a direct current field in a medium of constant specific resistance. The morphogenetic state produced by a given current density was independent of exposure time to a considerable degree, indicating that the control process was governed by the intensity factor (potential gradient) of the electrical energy, rather than by the capacity factor (current density) or the total electrical work.

The problem of whether the potential gradient or the current density is the independent variable in the effect of the electric field on growth direction, rate, or quality has not been attacked experimentally, although several investigators have expressed the belief that the potential gradient is the controlling factor (for literature see Evers, '47; Rosene, '47). Strong inferential evidence was presented by Lund ('25), who showed the threshold potential fall across the ecto-endoderm layer producing inhibition of growth toward the cathode in regenerating Obelia internodes to be equal to or slightly greater than the inherent potential, and opposite in sign. In Obelia inhibition is the immediate precursor of electrical control of differentiation.

The problem is of more than formal interest, since its solution decides between alternative classes of molecular events

which may constitute the electrical work performed, and between alternative implied relationships on the biological level. The electrical energy expended in a direct current circuit containing an aqueous element may produce (1) transport of material, (2) change of valence of material, (3) counter electromotive force, (4) dipole orientation, (5) dielectric strain, and (6) heat of overcoming resistance. Forms (1) to (5) possess characteristic magnitudes which are exclusive functions of either potential fall or current; electrical energy, of course, is a function of both. The heat of overcoming resistance need not be considered, since its generation would lack the polar quality necessary to a morphogenetic control agent. Moreover, in the majority of published experiments, the temperature rise would have been negligible had all the electrical energy appeared as heat (0.001 cal./sec./cm^3 maximal for reversal of polarity in Dugesia). Dielectric strain can probably also be ignored for the present, although in the form of the piezoelectric effect it has been suggested as a provisional explanation of inductance in cell membranes (Cole, '41).

If the current density were the critical determinant of the morphogenetic effect, the electrical work would take the form of (1) electrolytic and electrophoretic transport of charged material, and/or, if intracellular "electrodes" were present, (2) total quantity or rate of oxidation or reduction. Morphogenetic control would be established by a constant rate of movement of ions to, or creation at, sites of action, or by alteration of specific ionic balance due to differences in ion mobility. The essential process might be the ionic exchange between the organism and the medium, implying normal growth control by an environmental agent. As a corollary it would be anticipated that specific electrolyte mixtures would exert control in the absence of an external electric field.

If the potential gradient were the decisive factor, the electrical work would be some combination of forms (2) to (4) above. (2) A gradually increasing voltage applied to a complex solution between suitable electrodes will produce valence change of each electrochemically reversible constituent over

a narrow range of potential difference between electrode and electrolyte centered about its standard potential. If intracellular electrodes were present, morphogenetic control at constant potential fall would involve production of some necessary molecular entity (or entities) through oxidation or reduction at a critical energy level, chemical specificity rather than total quantity or rate of production being the distinguishing feature. (3) Development of counter electromotive force would modify preexisting patterns of cellular potential differences and establish a new electropolarity. Morphogenetic control would be effected through polar alteration of the cellular processes normally producing the potential differences, and would involve cellular metabolism to the extent and in the manner permitted by the nature and relationships of those processes. (4) The degree of orientation of a dipole with respect to field direction against the forces restoring random distribution is a function of the potential gradient and the dipole moment. As a control process orientation of some molecular species might be critical for inclusion in organized structure or participation in enzymatic or other function (cf. Lund, '47, p. 288).

The present experiments provide a test of whether the potential gradient or the current density is the decisive factor in the control of polarity of regenerating *Dugesia tigrina*. Control of growth quality by the field is the sole morphogenetic action determined; alterations of growth direction and growth rate, if present, are integrated into the differentiation process.

MATERIAL AND METHOD

The details of preparation of material, apparatus, and procedure previously given (Marsh and Beams, '52) apply to the present experiments with the modifications below.

Animals were divided into equal thirds after removal of head and tail, yielding pieces of from 3 to 4 mm length. Each piece was marked with a slit in the anterior end and

allowed to heal for an average of 12 hours before imbedding in 2% agar. All pieces were oriented with their anterior ends to the anode. Exposure time (120 hours), chamber position, specific resistance of medium, and total current were constant throughout a run. The temperature of the medium was maintained at $22 \pm 2°C$. Media with the specific resistances at 22°C. shown in table 2 were prepared by appropriate mixture of distilled water, tap water, and Ringer's solution. The data at 1010 ohm-cm in tables 1 and 2 are taken from the previous paper (Marsh and Beams, '52) and corrected to 22°C.

Preliminary experiments were run with the 2352 ohm-cm medium to determine the effective range of potential gradients necessary to produce 4 of the morphogenetic effects previously described: (1) regressive bipolarity, in which head structures and behavior appearing in the original posterior end disappeared subsequent to exposure; (2) permanent bipolarity; (3) progressive bipolarity, in which head characteristics residual in the original anterior end subsequently disappeared; and (4) reversal of polarity. Subsequently, for each medium, the pieces were set in the chamber at positions having potential gradients falling within this range. Current densities in $\mu a/mm^2$ were calculated from the chamber dimensions and the total current. The potential gradient in mv/mm is $0.01 \times$ current density $\times$ specific resistance.

As before (Marsh and Beams, '52), curvature of the isopotential lines in the chamber was overlooked. However, in the present experiments the number of pieces mounted in a single run averaged 17, with a maximum of 30. Thus, the entire body of data was obtained from a narrow strip across the chamber center where the error due to curvature did not exceed 2%. This circumstance is largely responsible for the smaller range of variation of the values of the potential gradient shown in the last line of table 1, compared to those for the 1010 ohm-cm medium.

RESULTS

In the preliminary tests with the 2352 ohm-cm medium the potential gradients producing the 4 morphogenetic effects agreed with those previously found with the 1010 ohm-cm medium (Marsh and Beams, '52). This was also the case for subsequent experiments with this medium and three others of higher specific resistance in which the animals survived for the duration of the experiments (table 1). Exceptions, excluded from the table, were 9 pieces which turned freely during exposure and 6 pieces in the 7095 ohm-cm medium which developed normally at a mean potential gradient of 180.8 mv/mm, a value indistinguishable from the group mean for regressive bipolarity in table 1. No differences could be distinguished in rate of regeneration or in morphogenetic state between regenerants in the 4 media and those previously described for the 1010 ohm-cm medium, nor did they differ from control animals save in those characteristics implicit in the bipolar condition.

Table 1 summarizes the effects of the electric field upon the regeneration axis of the 94 pieces surviving out of 513 mounted in media of 2352 to 12350 ohm-cm, together with the appropriate data at 1010 ohm-cm from the previous paper. For each value of the specific resistance shown in the left-hand column, the mean potential gradient is recorded for the number of regenerants showing each of the 4 morphogenetic effects. The mean current density for each group is shown in parenthesis beneath the potential gradient. The mean potential gradients at which a given morphogenetic effect was found are approximately constant and are independent of the specific resistance of the medium over a little more than a 12-fold range. The corresponding current densities diminish over the same range as the specific resistance increases.

Beneath the table is given the mean potential gradient and standard error of the mean for each of the morphogenetic states, calculated from the individual data. Each mean is significantly different from the others at the 0.0001 confidence

TABLE 1

Potential gradients producing 4 morphogenetic states in anodally oriented regenerating Dugesia in media of different specific resistance. *Current densities (μa/mm²) in parenthesis; 5 days' exposure. Range (1): 1010 ohm-cm; range (2): 2352 to 12350 ohm-cm*

SPECIFIC RESISTANCE IN OHM-CM	MORPHOGENETIC EFFECT							
	Regressive bipolarity		Permanent bipolarity		Progressive bipolarity		Reversal of axis	
	mv/mm	Number	mv/mm	Number	mv/mm	Number	mv/mm	Number
1010	183.5 (18.18)	7	198.3 (19.65)	21	205.2 (20.63)	11	222.7 (22.06)	14
2352	178.8 (7.45)	8	194.6 (8.03)	17	212.4 (8.84)	7	232.7 (9.73)	14
7095			188.4 (2.6)	8	201.0 (2.78)	2	227.5 (3.07)	8
10,390			190.3 (1.79)	8	203.0 (1.91)	1	219.0 (2.06)	7
12,350	183.6 (1.46)	3	191.5 (1.52)	7			224.0 (1.79)	4
Mean ± S.E.	181.5 ± 1.9	18	194.2 ± 1.54	61	206.7 ± 2.37	21	228.2 ± 2.91	47
Range (1)	169.4–195.6		174.0–233.7		190.5–237.3		195.0–320.0	
(2)	169.5–192.5		185.0–210.5		201.0–217.0		205.0–242.0	

level or better. The improved reliability of the means for the entire population compared to those previously reported for the 1010 ohm-cm medium alone (Marsh and Beams, '52) is in part due to the larger numbers of regenerants and in part to the smaller range of variation of the individual gradients found in the present experiments. Range (1) at the foot of the table is from the 1010 ohm-cm medium; the narrower range (2) is from the present experiments.

Four other media were tested, one below and three above the range of specific resistances in table 1. No animals survived exposure to the current for longer than 33 hours (15,550 ohm-cm medium). The per cent survival is shown in table 2, 4th column, for the specific resistances appearing in the first column. The data for 1010 ohm-cm is taken from the first paper. Per cent survival rises from zero at 469 ohm-cm through a variable course to an apparent maximum at 12,350 ohm-cm and descends abruptly to zero above this value. The populations tested were numerically disparate, and it is to be presumed that the high and low resistance points at which mortality becomes complete and the position of the maximum are approximate only. The last column in table 2 contains the average survival time in hours, which varies in a manner similar to the per cent survival. The averages for the 5 media in which regeneration occurred are made indeterminate by the arbitrary maxima set by the duration of exposure; except for nutritional difficulties there is no certain limit to survival time.

Mortality was greatest for midpieces and least for posterior pieces, with anterior pieces resembling the whole population. At a given specific resistance mortality showed no consistent relation to potential gradient (or current). In these respects the mortality behavior in the media of higher specific resistance resembles that in the 1010 ohm-cm medium previously reported (Marsh and Beams, '52); reasons were given there for believing the current to operate as an indifferent stimulus in producing death. For the entire set of media shown in table 2, however, the field operates as a lim-

iting factor for survival. *Dugesia tigrina* will regenerate normally in the absence of an electric field in all the media of table 2, although mortality is higher in the 469 ohm-cm medium due to failure of wound closure, and in the 51,100 ohm-cm medium. In the 469 ohm-cm medium reduction of field strength in one experiment to approximately one-half the range given in table 1 resulted in a survival of 14% (all normal regenerants). It appears reasonable to conclude that at or below some critical specific resistance the current drives ions into the regenerating piece faster than they can be expelled, while

TABLE 2

Survival of regenerating Dugesia anodally oriented in media of different specific resistance

OHM-CM SPECIFIC RESISTANCE	NUMBER MOUNTED	NUMBER SURVIVING	PER CENT SURVIVAL	HOURS AVERAGE SURVIVAL
469	60	0	0.0	2.5
1010	446	61	13.7	37.6
2352	334	53	15.8	28.2
7095	81	25	30.8	91.5
10,390	67	17	25.3	59.1
12,350	31	14	45.1	87.7
15,550	51	0	0.0	24.2
24,250	30	0	0.0	5.3
51,100	105	0	0.0	2.8

above some critical resistance the current drives out ions into the medium faster than they can be replaced, the ion excess or deficiency reaching instability levels. Evidence will be presented in a later paper consistent with the view that the internal ionic concentration is less than that of the external medium below a specific resistance of the latter of about 1600 ohm-cm and greater than that of the medium above this value. Since the per cent survival shows no direct relation to either potential gradient or current density, permeability changes induced by the field must play at most a minor role.

DISCUSSION

The results demonstrate that the potential fall across the regenerating piece is the independent variable in the effect of the electric field upon determination of the polar axis in *Dugesia tigrina*. Of itself, however, the demonstration will not support the inferences outlined in the Introduction, for if the internal resistance remained fixed as the external resistance varied, the constant potential gradient would produce a constant internal current. It will be shown in the following paper that the internal resistance does vary with the specific resistance of the medium, although to a lesser extent. It is assumed that the relation involves both body fluids and cells. Any generalization of the results obtained with Dugesia also implicitly assumes that other morphogenetic systems and other growth qualities possess analogous electrical characteristics.

In controlling axial polarity in Dugesia the applied electric field operates as a morphogenetic field and conforms to all the elements of the morphogenetic field concept (Weiss, '39). Since the latter, in its physical aspect, must have the characteristics of a force field, constancy of the controlling potential gradients may be considered as verification of a predictable relation. It is also a necessary prerequisite to identification of self-generated electric fields as the physical counterpart of the morphogenetic field, as in Lund's ('25, '28, '47) theory of cell correlation: that the patterned discharge of self-generated electrical energy through the conducting tissue fluids and cells acts as a naturally controlling complex electrical field in morphogenesis, and in Burr's ('32; Burr and Northrop, '35) electrodynamic theory of development. It may be noted here that the implications of control by constant potential gradient are all consistent with the self-contained, self-controlled nature of embryonic processes; those of control by constant current are not, or are so only under limited conditions.

Constancy of the potential fall excludes as mechanisms of control the extensive properties of the electric field, both pri-

mary and derived, associated with the magnitude of current flow, as developed in the Introduction, and limits those mechanisms to the intensive properties of valence change at critical energy levels, counter electromotive force, and dipole orientation. This will be true whether the field is conceived to operate upon substrate materials, catalysts, metabolism as a whole, or special control mechanisms such as organizers and evocators. Polar transport of evocators by the field would be inadequate as a single control mechanism, for their local supply would then be a function of current density as with any other ion. In addition the field must control their nature and formation, or tissue competence, or both. Some evidence exists (Lund, '47, Sec. IV) that this is also true of the presumably simpler electrical control of growth orientation in plant structures, wherein no test of whether the potential gradient is the independent variable has been made.

The possibilities of electrical control by orientation of dipoles is at present completely speculative, although it is known that several proteins in vitro possess characteristic dipole moments (Bull, '45, p. 70). Control through valence change at critical energy levels would necessitate the existence of cellular electrodes, for which there is no evidence save that implicit in Lund's oxidation-reduction theory of bioelectric potentials and the body of supporting experimental fact (Lund, '28; Marsh, '35; Rosene, '47). Control by establishment of a new electropolarity through development of a pattern of cellular counter electromotive forces specific for each morphogenetic state would be in harmony with the extensive data showing specific, self-generated electropolarity to accompany polar growth in a variety of living systems (Lund, '47). It is not not known whether the electropolarity imposed by an external electric field in a controlled system imitates in detail the natural electropolarity in the undisturbed system arriving at the same morphogenetic state.

Electrical control through counter electromotive force must extend through the metabolic mechanisms of establishment of cellular electromotive force to control of the metabolic

events of morphogenesis. If the electromotive system is of the type of the modified diffusion potential involving inorganic ions of the cell and medium, the connections must lie in the processes of ion accumulation, exclusion, and exchange, control of cell permeability, and relation of specific ions to enzyme activity and structural stability. The latter set of processes appear to be negated by the constant potential relationship, which will produce constant ion ratios across phase boundaries with diminishing absolute concentrations as the specific resistance of the medium rises, rather than the constant concentrations demanded by control by internal ionic mixtures. The relations of accumulation, exchange, and permeability to metabolism appear at present too limited in scope, number, and direction to constitute the basis for a theory of control.

If the electromotive system is conceived to be an oxidation-reduction couple generated in the course of normal respiration in accordance with Lund's theory, controlled valence change, establishment of specific electropolarity, and polar control of metabolism by an external field could be effected through the same mechanisms. The electrical characteristics of cells and tissues revealed by study of counter electromotive force (Hoyt, '47; Marsh, '30) and impedance (Cole and Curtis, '36, '44) resemble those of polarizable electrodes rather than modified diffusion potential systems or static capacities, although still other interpretations are possible. Lund's theory possesses the advantages of versatility, comprehensiveness, and adequacy to unify diverse areas of information. It possesses the disadvantages of unidentified electromotive materials and no demonstrated mechanism of electron transport. The modified diffusion potential theory possesses the advantages of demonstrable mechanism of establishment and the disadvantages of inadequacy of connecting relationships to metabolism and lack of comprehensive form. The precision and complexity of electrical control of polarity in Dugesia may be considered to be evidence for a direct and intimate influence of an applied electric field upon the course and

direction of cellular metabolism and an argument for the existence of cellular electrodes. In any event these considerations emphasize the desirability of investigation of the mechanism of development of inherent cellular electromotive force and other electrical characteristics upon as broad a basis as possible.

SUMMARY

1. Cut pieces of *Dugesia tigrina* were exposed to direct current for 5 days at 22 ± 2°C. in 8 media of specific resistance from 469 to 51,100 ohm-cm with their original anterior ends oriented to the anode.

2. The range of field strengths was that previously found to produce regressive, permanent, and progressive bipolar regenerants and reversal of the polar axis.

3. Animals survived longer than 33 hours only in media of 2352, 7095, 10390, and 12350 ohm-cm.

4. When combined with data at 1010 ohm-cm previously reported the results show that the potential gradients necessary to produce the 4 morphogenetic states above were constant and independent of the specific resistance of the medium over more than a 12-fold range.

5. The electrical work of control cannot be attributed to the rate or quantity of charge or material transported, the rate or quantity of valence change, or the total electrical work expended by the field. The work of control must be some combination of valence change at critical energy levels, development of counter electromotive force, and orientation of dipoles.

LITERATURE CITED

Brachet, J. 1950 Chemical embryology. Intersc. Publ., Inc., New York.

Bull, H. B. 1945 Physical Biochemistry. John Wiley and Sons, New York.

Burr, H. S. 1932 An electro-dynamic theory of development suggested by studies of proliferation rates in the brain of Amblystoma. J. Comp. Neur., *56:* 347–371.

Burr, H. S., and F. S. C. Northrop 1935 The electro-dynamic theory of life. Quart. Rev. Biol., *10:* 322–333.

Cole, K. S. 1941 Rectification and inductance in the squid giant axon. J. Gen. Physiol., *25:* 29–51.

COLE, K. S., AND H. J. CURTIS 1936 Electric impedance of nerve and muscle. Cold. Spr. Harb. Symp. Quant. Biol., IV: 73–89.

———— 1944 Electrical physiology; electrical resistance and impedance of cells and tissues. Pages 344–348 in O. Glaser, Medical Physics. Year Book Publ., Chicago.

EVERS, J. 1947 Bioelectric fields and growth, pp. 151–161. Univ. of Texas Press.

HOYT, R. C. Counter-emfs in the onion root. J. Cell. and Comp. Physiol., *29:* 109–130.

LUND, E. J. 1925 V. The nature of the control of organic polarity by the electric current. J. Exp. Zool., *41:* 155–190.

———— 1928 Relation between continuous bioelectric currents and cell respiration. II. J. Exp. Zool., *51:* 265–290.

LUND, E. J. ET AL. 1947 Bioelectric fields and growth. Univ. of Texas Press.

MARSH, G. 1930 The effect of applied electric currents on inherent cellular E.M.F. and its possible significance in cell correlation. Protoplasma, *11:* 447–474.

———— 1935 Kinetics of an intracellular system for respiration and bioelectric potential at flux equilibrium. Plant Physiol., *10:* 681–697.

MARSH, G., AND H. W. BEAMS 1952 I. Relation of axial polarity to field strength. J. Cell. and Comp. Physiol., *39:* 191–213.

NEEDHAM, J. 1931 Chemical embryology. Cambridge Univ. Press.

ROSENE, H. 1947 Bioelectric fields and growth, pp. 301–391. Univ. of Texas Press.

WEISS, P. 1939 Principles of development. Henry Holt and Co., New York.

THE IMPAIRMENT OF VISUAL CELL STRUCTURE BY IODOACETATE

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SIXTEEN FIGURES

In a previous communication iodoacetate has been shown to produce immediate and selective effects on the function of the mammalian retina when administered intravenously (Noell, '51). It abolished the responsiveness to illumination of almost all visual cells within minutes and for several hours. From the character of the electrical changes which accompanied the effect it appeared that excitatory processes within the visual cells were primarily affected. Moreover, it seemed that the effect was caused by the dependence of visual cell processes on glycolytic reactions. In initiating the present study, the slow recovery was taken to indicate that the impairment of sensory functions might be associated with structural changes evoked either by a direct effect of iodoacetate on the general metabolism of the visual cell (e.g., inhibition of glycolysis) or, indirectly, by the loss of a special (sensory) function, which, in turn, influenced the structural organization of the cell. In any event, if iodoacetate produced an irreversible effect on retinal function and if the conclusion as to the site of this effect was correct, the iodoacetate treated retina should offer an exceptional opportunity for the study of numerous problems concerning the relationship between retinal function and visual cell structure. Toward this end, retinae, exposed to the effects of the agent, were studied microscopically in conjunction with a determination of their electrophysicological properties. The histological findings described below are thought to provide the basis for the application of the principle outlined.

EXPERIMENTAL PROCEDURE

Albino rabbits, cats and two monkeys (Macaca mulatta) were treated by intravenous administration of iodoacetic acid in amounts sufficient to produce irreversible impairment of retinal function. After a variable length of time (12 hours to 9 weeks) their retinae were explored bioelectrically and subsequently removed for histological examination. The resting potential across the eye-bulb and the electroretinogram were measured with Ag-AgCl electrodes, one electrode connected by a Ringer fluid bridge with the cornea, the other connected similarly with the tissue behind the eye-bulb. In rabbits, the retina of one eye was examined further by Granit's microelectrode technique (Granit, '47) and the responsiveness of one optic nerve to direct electrical stimuli was tested by recording the responses of the controlateral striate area. One or both eyes then were removed and immediately immersed in Zenker's solution. Paraffin sections of 6 μ thickness were cut along the vertical meridian in rabbits and through the horizontal meridian in cats and monkeys. The slides were stained routinely with hematoxylin-eosin; in most cases the Heidenhain-phloxine and the Mallory trichrome stain were also employed.

The iodoacetate solutions were prepared from recrystallized iodoacetic acid which was neutralized with NaOH immediately before use. In rabbits, the initial dose was generally 20 mg iodoacetic acid per kilogram bodyweight. A second injection of 15 to 20 mg/kg followed 6 to 8 hours later. Thereafter, the treatment was individualized, depending on the recovery of blink and pupillary reflexes and the general status of the animal. The treatment was either stopped or the injection of 15 to 20 mg/kg was repeated once or twice during the following 24 hours. In cats, two injections of 12 mg/kg of iodoacetic acid within 24 hours proved to be as effective as the larger doses used in rabbits. In the monkey several injections of 20 to 40 mg/kg were employed to obtain irreversible effects.

RESULTS

1. Status of retina three weeks after poisoning. Ten rabbits were studied 18 to 22 days after the administration of the poison. Twenty milligrams of sodium iodoacetate per kilogram bodyweight had been injected intravenously 3 to 4 times during a period of 24 hours. In all animals the blink-reflex disappeared after the injections whereas a minimal response of the pupils to strong illumination was not abolished permanently. The animals were in good health for almost the entire period of observation although initially some had exhibited decreased activity for one or two days. When tested at the end of the period of observation it was impossible to evoke any trace of an electroretinogram in 9 animals whereas one eye of one rabbit showed a barely measurable response of about 5 μv. Examination of the retinae by the microelectrode technique revealed (after long searching) the presence of spontaneously active elements, the number of which was considerably reduced compared with normal rabbits examined by the same technique. These spontaneously active elements were difficult to isolate and were less susceptible to the pressure of the electrode than is normally experienced. Responses of these elements to illumination were generally absent; occasionally a group of elements was found which either responded with inhibition or showed a slightly increased discharge rate shortly after the abrupt cessation of illumination. Direct electrical stimulation of the optic nerve produced normal responses of the striate area and changes in the excitability of the nerve were not apparent. A steady potential across the eye-bulb ranging between 2 and 2.7 mv. was present in 5 animals, in the others it was .5 mv. or less. Employing the same technique for the preparation of the eye and its immobilization (curare) normal values for the steady potential ranged between 2 and 4.5 mv.

Microscopically, the retinae of these rabbits showed widespread disappearance of visual cells (plate 1 A, B). With the exception of a small area along their anterior border

(ora serrata) and another around the optic nerve, the retinae were devoid of almost all visual cells; outer and inner segments had disappeared as had the outer nuclear layer. Occasionally, a single pyknotic nucleus could be found in the space between inner nuclear layer and outer limiting membrane which normally is occupied by about 4 rows of visual cell nuclei. Now this space was reduced in width and contained only the outer plexiform layer and few newly-grown Müller fibers. In contrast, the inner layers of the retina were generally well preserved (plate 1, A). Sections through the optic nerve showed no difference from normal. In several rabbits iodoacetate also had little or no effect on the non-nervous tissues (plate 1, A), in others the loss of the visual cells was associated with the disappearance of the pigment epithelium in the mid-periphery of the retina and with marked reactions of glia elements (plate 1, B).

Essentially the same histological changes as in albino rabbits were found in cats three weeks after the administration of iodoacetate. There was a similar widespread disappearance of the visual cells while the bipolar and the ganglion cells were preserved (plate 2, A). The pigment epithelium had vanished locally and glia had proliferated. In addition, there was intraretinal pigment accumulation (plate 2, B). The retinal vessels which in the cat as in primates vascularize the inner layers, became markedly narrowed within the first two weeks after iodoacetate administration — apparently in response to the disappearance of the visual cells and the thus reduced need for a retinal vascular system in addition to the choriocapillaris.

2. Local differences in resistance of rod population. In all retinae of rabbits and cats, the visual cells near the ora serrata and near the optic nerve entrance were the most resistant cells of the entire population. At the ora serrata of rabbits, for instance, outer nuclei were preserved three weeks after the treatment for distances ranging from a fraction of a millimeter to 2 mm when a total of 60 to 80 mg iodoacetate per kilogram had been injected (plate 1, C). In gen-

eral, the higher the dosage of iodoacetate the smaller was the number of preserved nuclei and the shorter the segment of the retina which contained visual cells or their remnants. Along such a segment the number of outer nuclei decreased almost exponentially to zero with increasing distance from the ora serrata. The nuclei either stained normally or were in various stages of degeneration; pyknotic ones occupied the border zone to the mid-periphery. Similar conditions prevailed around the optic nerve entrance and, in the rabbit, for a short distance along the horizontal band of myelinated nerve fibers.

Outside these areas, variations in the extent of visual cell disappearance occurred over a narrow range of low dosages. In the few rabbits in which such variations were encountered maximal damage was located in the center of the retina, ventral to the optic nerve entrance and in a short segment about halfway between optic nerve and ora serrata. In these areas, all visual cells had vanished occasionally while in others their number was reduced to half and none had disappeared around the ora serrata. Illustrating the low probability of its occurrence, unequal distribution of the lesion was associated with great differences in the histological appearance of both retinae of the same animal. Thus, whereas one eye had lost less than half its visual cell population in the described fashion, the other eye could be devoid of almost all visual cells. It seems, therefore, that the metabolic property of the visual cells which is affected by iodoacetate is rather uniformly distributed over the rod cell population. The observed differences in resistance might be caused by unequal distribution of the poison or by other factors not conditioned by the visual cells themselves.

3. Dosages and the time course of rod cell changes. In general, a single injection of iodoacetate (20 mg/kg in the rabbit, 12–15 mg/kg in the cat) abolished the electroretinogram and other manifestations of visual cell function only temporarily. The electroretinogram (b-wave) recovered to more than half its normal size within 5 to 6 hours and had

regained almost its normal form within the next day. Irreversible effects, however, were produced when the injection was repeated once or twice. Such division of dose was necessary to prevent the overwhelming (general) toxicity of a single dose which usually was lethal when 30 to 40 mg kg, were employed. Nevertheless, the majority of rabbits tolerated three injections of 20 mg/kg bodyweight administered within 24 hours. Cats were more susceptible and two injections of 15 mg/kg were followed by severe central nervous, intestinal and renal symptoms which lasted at least several days. These doses were, however, above the amount necessary for the gross impairment of visual function. In the monkey, on the other hand, both the retinal and the general toxicity of the agent was less than in cat and rabbit, and a single injection of 30 mg/kg was almost ineffective.

The rapidity with which iodoacetate abolished the electroretinogram was reflected by its histological effects on the retina. Pyknosis of the majority of rod cell nuclei was present in less than 24 hours after the second or third injection indicating that the death of the visual cells occurred almost immediately after the abolition of their sensory functions. There was likewise a rapid elimination of the dead cells and in the rabbit 10 days sufficed for the removal of almost all visual cell remnants. When the effect of the poison was of the described rapidity all organelles of the rod cells were simultaneously affected. Even within the first day after the effective injection it was impossible to decide whether there were any differences in susceptibility. Outer and inner segments were swollen and the nuclear chromatin was in a pyknotic state. However, when the effect of the poison became manifest slowly as it did in the areas around the ora serrata, the outer sement was the first to disappear while a deformed inner segment — appearing as a short and thick stump — was preserved for some time together with a normally staining nucleus, which in turn survived longer than the inner segment. The progress of degeneration was of such slowness in these areas that it took more than 9 weeks until the visual

cells had either disappeared completely or survived by maintaining normal forms. Comparing the retinae from different rabbits treated in the same manner but removed at different times (2 to 9 weeks after the injections) it appeared that not only the process of degeneration, once started, was very slow in these areas but that also its beginning was delayed with respect to the bulk of the rod cell population. At an early time of examination, a greater number of retinae showed well preserved visual cells in these areas than several weeks later.

From the above description, the effectiveness of iodoacetate appears to cover the range from a transient functional impairment to the death of the visual cells. Qualifying the first mentioned phenomenon there were indications that transient functional impairment may be associated with permanent but latent changes in the cells. Most illustrative in this respect was the case of a cat (1–35) which had been treated by one injection of 22 mg/kg of iodoacetic acid. Immediately after the injection visual performance was abolished for about 20 minutes and 24 hours later both eyes again seemed to function perfectly. They did so for an observation period of three weeks at the end of which time one eye was enucleated during urethane anesthesia. When recovery from anesthesia had occurred the remaining eye was found to have become blind. Histologically, the eye removed three weeks after iodoacetate treatment revealed a perfectly normal retina; the other eye, however, removed three weeks later showed almost complete loss of outer and inner segments, all outer nuclei being pyknotic. Since urethane alone in the doses used did not produce permanent visual effects it seems that it made manifest a change which had remained latent after the administration of iodoacetate. Repeating the experiments in the same fashion in other cats similar results were obtained. The effectiveness of urethane weeks after one injection of iodoacetate was, however, not restricted to the visual cells alone; likewise lasting central nervous symptoms appeared reminiscent of those which were experienced after repeated injections of iodoacetate.

4. Cone cells. The destructive effect of iodoacetate on the visual cells differed in cone and rod population with respect to time course and intensity, as demonstrated by tissue slides from a monkey's retina (plates 3, 4). The monkey has been treated by iodoacetic acid over a period of 12 days, during which time a total of 300 mg (i.e., 100 mg per kilogram bodyweight) had been injected. The injections had been entirely ineffective with respect to health and behavior of the monkey, and even his visual abilities did not appear greatly impaired when tested by his skill to catch food. The pupils, however, responded less intensely to illumination and were moderately dilated. Five weeks after the last injection the retina was examined electrically during Dial anesthesia. The electroretinogram was normally configurated, a- and b-waves being in the normal proportion to each other, whereas the total size of the electroretinogram was about 100μv. compared with 200 to 250 μv. in normal monkeys under the same experimental conditions. Microscopically, the retina was more severely damaged than was expected. An area amounting to about 65% of the total retinal arc was devoid of all rod cells, except for some pyknotic nuclei scattered at random. The well preserved area of the retinal arc (with regard to rod cells) was located peripherally, close to the ora serrata. It contained rod and cone cells with slightly swollen or normal organelles. Throughout the retina, the bipolar cells, ganglion cells, and optic fibers appeared in normal number and with normal staining characteristics in accordance with the results obtained in rabbit and cat.

Most remarkable was the appearance of the cone cells. In the center of fovea they exhibited entirely normal features, including all organelles (plate 3, A). Parafoveal, however, their appearance changed and most remarkably this change became continuously more severe as the distance from the center of the fovea increased (plate 3, B–D). First, there was swelling of the inner segments (including the ellipsoid body) resulting in their broadening. This change became progressively more marked towards the periphery, causing

the cones to appear in a closed row for about 4 mm around the fovea. The swelling was closely followed by longitudinal "shrinkage" of the proximal parts of the segment, and the height of the inner segments continuously decreased until the (deformed) ellipsoid body rested upon or levelled off with the external limiting membrane (plate 3, D; 4, B). The outer segment likewise underwent a process of swelling and internal and external deformation. It finally formed a lightly staining cup covering the broadened ellipsoid body when the height of the inner segment has been reduced to about a third (plate 3, C). The nucleus and the perinuclear cytoplasm survived these changes and over the whole retina not a single degenerated cone nucleus could be discovered nor was there any evidence that cone nuclei had disappeared. Thus, after the cone cells had lost all special organelles they appeared as a row of simple epithelium (plate 4, A), calling to mind, rightly or wrongly, the ancestral history of the visual cells which, according to Studnička (1898), descend from ependymal cells.

The most resistant special organelle of the cone cells was the ellipsoid body. Apparently, due to shrinkage of the plasmatic bridge between the perinuclear space and the inner segment, the ellipsoid body was finally forced to cross the external limiting membrane and to take a position close to the nucleus where it either persisted or underwent dissolution (plate 4, A). Since considerable forces must have developed to condition these positional changes, it might be possible that shortening of the myoid was the responsible event, the myoid being the structure which in lower vertebrates produces changes in cone position in response to illumination (Dittler, '29). Many ellipsoid bodies of rod cells in rabbit and cat likewise became located inside the outer limiting membrane during the early course of degeneration. These ellipsoid bodies vanished before the nuclei had disappeared.

In rabbits and cats, as in many other vertebrates having an abundant rod population, cone cells can be recognized by

the organization of their nuclear chromatin which forms a reticulum of fine granules in contrast to the rods where the chromatin is collected in two or more close clumps (Walls, '42). In addition, there are also some differences in the shape and size of the nucleus, the cone nucleus being large and round, the rod nucleus smaller and oval. Analyzing the retinae of cats and rabbits according to these properties, it was found that in early stages of degeneration (4 to 10 days) the outermost row of the outer nuclear layer contained a great number of large and round nuclei with reticular chromatin, whereas almost all other nuclei of the layer were pyknotic or had disappeared (plate 4, D). This finding was most conspicious in cats. Some of these "cone-like" nuclei were connected with short and thick inner segments but none of these segments was reminiscent of a cone. Moreover, the number of these cone-like nuclei was unreasonably high and could not be accounted for by the examination of normal retinae. It is, therefore, assumed that many of these nuclei represented degenerating rod nuclei which during the course of degeneration "regressed" to a (transient) cone-like appearance. Since it is the outermost row of the nuclear layer which shows this "regression" the phenomenon might be related to the ability of the rods and cones to transmute during the vertebrate evolution and to produce "intermediates." The vivid discussion of transmutation and "intermediates" by Walls ('42) tempts one to assume such a relationship.

5. Pigment cells, Müller cells and membranes. Changes in the framework of the retina occurred at a different plane of poisoning than that associated with the disappearance of the visual cells. Thus, the retinae of many rabbits, devoid of visual cells except for the areas around the ora serrata and the optic nerve, did not show more than minor reactions of the non-nervous tissues 2 to 9 weeks after treatment. There were a few Müller cells which had invaded the outer plexiform layer; the pigment epithelium formed a continuous, well preserved layer in loose contact with the outer limiting mem-

brane (plate 1, A). Fine glia fibers extended from or through the outer limiting membrane into the open space between the membrane and the epithelium wherever the retina had become detached during the histological preparation. In other cases, however, marked reactions of glia and pigment epithelium were encountered as early as 14 days after the injections. Iodoacetate had been administered in higher doses in these cases than in the majority of those in which major reactions of the supporting tissues were missing. Consequently, fewer visual cells were preserved around the ora serrata and the optic nerve, and fewer outer nuclei had survived at any time. The reactions of the supporting tissues were complex and those of the glia elements were closely associated with those of the pigment cells. Three weeks after the treatment they never involved the whole retina but were restricted to midperipheral areas. In these areas the pigment epithelium had disappeared; the outer limiting membrane had been perforated at one or several spots through which Müller cells had invaded the space between the outer limiting membrane and the choriod (plate 5). Once proliferating outside the membrane, Müller cells advanced also into spaces covered by still intact pigment epithelium; they then separated the membrane from the epithelium without breaking their continuity at first. With the further development of these changes, the pigment epithelium and outer limiting membrane disappeared extensively and Müller cells stratified the space between choriod and bipolar cells. In no instance, however, was the choriodal side of the lamina vitrea interrupted; it was finally the only continuous barrier left between retinal and choriodal structures. The inner layers of the retina were distorted, reduced in width or totally atrophied several weeks after the pigment epithelium had vanished over large areas. Bipolar cells and ganglion cells, however, almost invariably were preserved even 9 weeks after the treatment (and the consequent death of the visual cell population) when the pigment epithelium had survived.

In cats the changes described above were accompanied by the appearance within the retina of pigment clumps of varying size, some as large as big ganglion cells (plate 2, B). The clumps were scattered over all retinal layers and in regions surrounding large retinal vessels. The corresponding pigment epithelium had disappeared, or had lost its pigment. In the latter case it either appeared otherwise normal or had formed a double layer or was in an advanced state of degeneration (pyknosis). Intraretinal pigment deposition was almost absent in areas where the covering pigment epithelium did not normally contain pigment, i.e., in front of the tapetum cellulosum (plate 2, A), nor was it found in areas where the visual cells had not vanished and the pigment epithelium had been well preserved. Marked pigment deposition was restricted to the midperiphery, halfway between the ora serrata and the optic nerve. In the albino rabbits destruction of the epithelial layer was localized in about the same regions as in the cat, except for a preferential involvement of the ventral half of the retina.

In the monkey the pigment epithelium was a normal one in each section of the retina, the outer limiting membrane was intact and proliferation of Müller cells within the retina was not revealed. As described previously, the majority of rod cells had vanished, but all cone nuclei had apparently survived.

Early changes of the pigment cells included swelling and multiplication (4th to 10th day) and the appearance of cells containing two nuclei. Mitotic activity was not apparent.

The event initiating the reactions of the pigment epithelium was not revealed with certainty. It is, however, very improbable that its degeneration occurred in response to the destruction of the visual cells since the epithelium was well preserved in the majority of rabbits treated by a relatively low dose of iodoacetate despite the invariable disappearance of almost all visual cells. Products of visual cell degeneration are, therefore, very probably not the cause for the death of the pigment cells nor is there evidence that secondary

changes of the choriodal circulation could be responsible. It, thus, appears that iodoacetate affects directly three closely related elements of the mammalian retina: rod cells, cone cells and pigment epithelium, the rod cells being the most, the pigment epithelium the least, susceptible. The histogenic problems of pigment cell degeneration and glia reaction often have been discussed in relation to diseases of the retina (e.g., retinitis pigmentosa) which produce similar or identical histological changes as described here for the effects of iodoacetate. It is generally assumed (Leber, '16; Verhoeff, '31; Asher, '32; and Elwyn, '46) that the pigment cells proliferate and become ameboid in response to the stimulus of rod and cone degeneration and that they grow or wander into the retina where their pigment accumulates while the cells themselves undergo destruction. Though pigment deposition was an essential feature of our results (cat) evidence for the mechanism outlined was not revealed.

DISCUSSION

The preciseness with which iodoacetate affects the visual cells of the mammalian retina and the ease with which the consequences are measurable are barely surpassed by drug effects on unicellular organisms living in an artificial medium. This may be conditioned by the following circumstances. A given population of visual cells, rod cells or cone cells, is rather uniform with regard to structural properties; all individuals are of the same age; each individual is practically independent from the others, both anatomically and functionally. The population lives in a space which is almost free from other living matter, its medium maintained by diffusion from or to tissues enveloping the space. With respect to the whole organism, each individual serves but one purpose, the transformation of light into excitation, the efficiency of the underlying processes being readily measurable. Accordingly, if the population differs in one important property from others of the mammalian organism and if this difference makes it more susceptible to any one

agent, experimental conditions of great simplicity will prevail similar to those of *in vitro* studies with the additional advantage of measuring a specialized function.

The effects of iodoacetate may be subdivided into fast and slow ones, the former covering the range from reversible functional impairment to almost immediate death of the majority of the cells. Slow effects include two noteworthy features: (1) delayed manifestations of structural impairment and (2) regression to primitive cell forms. Regression was exhibited by a gradual loss of the specialized organelles — outer and inner segments — and a transient persistence of the nucleus and the perinuclear cytoplasm. This was best demonstrated by the cone cells, probably due to the fact that their greater resistance to the agent facilitated the appearance of graded responses. It was, however, likewise apparent for the rod cells. Assuming that iodoacetate affects the general metabolism of the cells, it follows that the maintenance of outer and inner segments requires greater efforts or closer to optimal conditions than that demanded by the nucleus and its surrounding. Conversely, loss or degeneration of outer and inner segments does not appear to be sufficient cause for the death of the visual cell within a short period of time (several weeks).

Delayed manifestation of the structural effects of iodoacetate became apparent though the design of the experimental procedure — attempting the production of immediate cell death — was most unfavorable for its discovery. Thus the phenomenon was not revealed by the majority of the visual cells but by those showing a greater resistance than the rest, i.e., rod cells around the ora serrata and cone cells around the fovea. The presence of well preserved or newly degenerating cells in these areas at time intervals of up to 5 weeks after the injections indicates that changes induced by iodoacetate may remain latent histologically for several weeks. There is reason to assume that during this latent period the sensory function of the cell is not abolished. For example, in the monkey vision must have been maintained

by the cones of the fovea which exhibited normal forms 5 weeks after the injections, whereas the parafoveal cones showed degeneration of increasing intensity the greater the distance from the center of the fovea. The change, however, from normal to abnormal appearance along a section through the fovea was so smooth that it must be interpreted as a change from latent to manifest damage of cell structure. Similarly, the described effect of urethane weeks after one injection of iodoacetate may be interpreted as the manifestation of a change in the cells which had remained latent functionally as well as anatomically after the administration of iodoacetate.

The most embarrassing feature of the effects of iodoacetate on the mammalian retina was the striking histological similarity with a certain group of human heredodegenerative diseases, generally referred to as retinitis pigmentosa. Great similarities between the effects of the agent and these diseases had been expected at the onset of the study since in both cases the visual cells were assumed to be affected primarily. The similarities, however, gained almost unbelievable proportions in view of the natural reluctance to consider a close relationship between chemically induced changes and a human disease of hereditary character. There is not a single detail in our findings which has not been described in the disease nor is there any essential feature of the disease which is not revealed by the experiments. To avoid repetition the following list of characteristics considered essential and identical in both cases may suffice: disappearance of visual cells; greater survival times of cone cells compared with rod cells; increasing intensity of cone cell changes with increasing distance from fovea (Stock cited by Leber, '16); greater resistance of visual cells around ora serrata versus midperiphery (Verhoeff, '31); preservation of inner nuclear layer and ganglion cells; disappearance of pigment cells; deposition of pigment within inner layers; preservation of pigment epithelium in areas where cone cell remnants have survived (Verhoeff, '31); type of Müller cell reactions (perforation of ex-

ternal limiting membrane, etc.); and intactness of the choriod. The great difference is in the time course with which the changes develop. Retinitis pigmentosa usually appears at the end of the first or during the second decade of life and results in practical blindness in the 30's to 50's, whereas iodoacetate is able to produce the same changes within several weeks. The hereditary character of the disease (generally recessive) has been established particularly by Nettleship ('07–'08). By general consensus (Leber, '16; Verhoeff, 31; and Elwyn, '46) retinitis pigmentosa is considered as a primary heredodegerative disease of the neuroepithelium, the reactions of pigment epithelium and glia, contrary to our interpretation of the iodoacetate effects, assumed to be secondary (non-specific) to the disappearance of the visual cells. Rapid development of visual cell degeneration on a hereditary basis has been described for a strain of rats by Bourne, Campbell and Tansley ('38). Attempts to produce the disease experimentally have failed in the past as have various proposals for treatment. Effects of strongly oxidizing agents on the retina, such as sodium iodate, have been interpreted by Sorsby ('41) as very similar to the disease and have led him to consider a disturbance in the ascorbic acid metabolism as the common factor. In accord with Homma ('35) and others it was found, however, that iodate produces a toxic chorioretinitis with primary involvement of the pigment epithelium (Noell, '52).

The selective effectiveness of iodoacetate on the visual cells of the mammalian retina must be conditioned by two properties; the permeability of these cells for the agent and the presence of a metabolic system susceptible to iodoacetate and very closely linked with the functioning of the cell. With respect to the latter property it will be considered first whether or not the enzyme system affected is incorporated into the general metabolism of the visual cells or is developed locally for the maintenance or performance of the specific sensory function. Wald and Brown ('51) recently revealed that sulfhydryl groups are involved directly in the

binding of carotenoid to protein in rhodopsin and that a sulfhydryl reagent (p-chloromercuribenzoate) is able to block the regeneration of rhodopsin after bleaching. Iodoacetate and iodoacetamide, however, did not exert such an effect *in vitro*. For the following it is also very improbable that it did so *in vivo* and that inhibition of visual purple regeneration was the primary cause of our observations. First, iodoacetate did not produce the same sequence of electroretinographic changes in rabbits as in cats. This is contrary to what might be expected if the agent inhibited one fundamental reaction of vision in a selective manner. Furthermore, nuclear degeneration after iodoacetate administration was so rapid that one cannot assume it to be secondary to a localized effect on the outer segment. In the previous study (Noell, '51), evidence was obtained to indicate that iodoacetate produced its immediate effect on visual cell function by the inhibition of glycolysis, the retina being well known for its high lactic acid production. This evidence was based mainly on a comparison of anoxia with iodoacetate poisoning which showed that the drug affected with least ease those components of the electroretinogram which responded rapidly to anoxia. It was furthermore found in rabbits that during an 8 minute period of ischemia shortly preceded by the intravenous injection of 20 to 25 mg iodoacetate per kilogram bodyweight, the accumulation of retinal lactic acid was reduced to about half the control measurements (Chinn and Noell, '51). There are no difficulties in the interpretation of the rapid histological effects of iodoacetate by assuming the same mechanism of action, i.e., reversible functional impairment and cell death to be the minimal and maximal level of action, respectively, both induced by the almost instantaneous reduction of cellular energetics beneath critical points. Two phenomenon of this study, however, seem to go beyond such an interpretation in its simplest form and make it necessary to take into account the natural complexity of intracellular reactions. The phenomenon of a latent period between the initial reversible effect of an

agent and the manifestation of irreversible cell damage, as it was observed for submaximal doses of iodoacetate, has been particularly emphasized for agents of assumed or proven nuclear effectiveness such as mustard gas (Auerbach, '49), carcinogenic substrates, e.g., benzanthracene (Berenblum, '47); lewisite (Danielli, '50). Furthermore, the study indicated very strongly that the metabolic property of visual cells and pigment epithelium which is responsible for the action of iodoacetate is distributed in the same fashion over these cell populations as that property which in humans determines cellular degeneration by an abnormality in the genetic pattern. To account for these peculiarities, the hypothesis is entertained that the carbohydrate metabolism of the visual cells depends on an intimate relation between a gene and a particular enzyme; the acute action of iodoacetate determined by enzyme inhibition, the delayed one by effects on the genetic control mechanism. Assuming some kind of interaction between enzyme and gene, the latter effect of iodoacetate may be induced not directly, but indirectly by the initial and transient enzyme inhibition inasmuch as this affects the energetics of the cell; it furthermore could become manifest (on cell structure) by influencing adversely the same enzyme system, the impairment of which constituted the initial change. The assumption of such a relation between a gene and the iodoacetate sensitive enzyme seems, at the present, best to account for the identical distribution and character of the histological changes under the three conditions outlined; first, after maximal doses of iodoacetate inducing immediate structural effects, second, after submaximal doses producing the effects with delay and third, in hereditary degeneration of visual cells and pigment epithelium the idea being that in all three cases one property, the degree of dependence on glycolysis, determines the distribution of the histological changes. Emphasis thus placed on one principal mechanism of action, the very narrowness of such view should not remain unstated. Since very probably iodoacetate produces its most striking biological effects by

combining with the thiol groups present in such systems (Barron and Singer, '45) numerous possibilities of attacking intracellular reactions are given ranging from actions on glycolytic and respiratory enzyme systems (Peters et al., '35) to reactions with essential nuclear proteins (Rapkine, '33). Its selective effect on visual cells and pigment epithelium while sparing all other elements of the retina though the agent had penetrated the tissue is, however, a strong argument for reasoning that one especially develepod property of the former is responsible for the observed effects.

SUMMARY

Examining microscopically the retinae of mammals (rabbit, cat, macaca mulatta) rod, cone, and pigment cells were found to be irreversibly affected by intravenous administration of iodoacetate. Depending on dosage and individual cell resistance the effects on the structural maintenance of the cells became either manifest immediately after the injections or with a delay of days or weeks after an initial, reversible impairment of functioning. Most susceptible was the rod cell population. It survived less than 24 hours and vanished almost completely within two weeks after injections. Bipolar cells and ganglion cells were primarily preserved. Rod cell resistance was greatest in areas close to the ora serrata and the optic nerve. If degeneration proceeded slowly it involved first the outer and inner segments, nuclear degeneration following with delay.

In the monkey, the cone cell population was found to be less readily affected by the agent than the rod cells. While the latter disappeared over the greater part of the retina, the most advanced change of cone cells was the loss of all specialized organelles. The severity of the structural impairment of the cone cells increased continuously with the distance from the center of the fovea. The most resistant specialized organelle of the cone cells was the ellipsoid body.

Degeneration of the pigment epithelium occurred when doses higher than necessary for the death of the visual cells

alone were employed. Disappearance of pigment cells was associated with glia proliferation, destruction of the outer limiting membrane and with intraretinal pigment accumulation.

The possible mechanism of the action of the agent on the retina is discussed. It is emphasized that its histological effects are in all essential features the same as described for heredodegenerative diseases of the neuroepithelium.

ACKNOWLEDGMENT

Miss Grace Paul from the Department of Pathology (Lt. Col. R. B. Lewis) prepared the histological sections of the eyes and Mr. Ivan Richardson from the Department of Medical Photography (Capt. H. R. White) the photographs. I desire to express to both my appreciation for their very kind and skilled cooperation.

LITERATURE CITED

Asher, K. 1932 Zur Histologie der Pigmentdegeneration der Netzhaut. Arch. f. Augenh., *106:* 585.

Auerbach, C. 1949 Chemical mutagenesis. Biol. Rev., *24:* 355.

Barron, E. S. G., and T. P. Singer 1945 Sulfhydryl enzymes in carbohydrate metabolism. J. Biol. Chem., *157:* 221.

Berenblum, I., and P. Shubik 1947 Brit. J. Cancer, *1:* 379.

Bourne, M. C., D. A. Campbell and K. Tansley 1938 Hereditary degeneration of the rat retina. Brit. J. Ophth., *22:* 613.

Chinn, H. I., and W. K. Noell 1951 Chemical changes in brain during anoxia. USAF SAM Project No. 21 23-011, Report No. 1.

Danielli, J. F. 1950 "Cell Physiology and Pharmacology." Elsevier Publishing Company, Inc., New York.

Dittler, R. 1929 Die objektiven Veranderungen der Netzhaut bei Belichtung. Handb. norm. path. Physiol., 12/2, I, 595.

Elwyn, H. 1946 "Diseases of the Retina." The Blakiston Co., Philadelphia.

Granit, R. 1947 "Sensory Mechanisms of the Retina." Oxford Univ. Press, London.

Homma, R. 1935 Experimentelle Untersuchungen ueber pathologische Secretions Vorgaenge des retinalen Pigmentepithels. Graefes Arch. f. Ophthalmol., *134:* 305.

Leber, Th. 1916 Die Krankheiten der Netzhaut. In Graefe-Saemisch: "Handbuch der gesamten Augenheilkunde." 2d ed., vol. 7, part 2, p. 1076. Wilhelm Engelmann, Leipzig.

NETTLESHIP, E. 1909 On some hereditary diseases of the eye — The Bowman Lecture. Tr. Ophth. Soc. U. Kingdom, *29:* 128.

NOELL, W. K. 1951 The effect of iodoacetate on the vertebrate retina. J. Cell. and Comp. Physiol., *37:* 283.

———— 1952 The effect of azide on the steady potential across the eye-bulb. Am. J. Physiol., in press.

PETERS, R. A., H. RYDIN AND R. H. ST. THOMPSON 1935 The relation of pyruvic acid in brain to certain tissue poisons. Bioch. J., *29:* 63.

RAPKINE, L. 1933 Sur l'action de l'acide monoiodoacétique sur les groupements sulfhydrilés liés aux protéines. Compt. rend Soc. Biol. Paris, *112:* 1294.

SORSBY, A. 1941a Experimental degeneration of the retina by sodium iodate. Brit. J. Ophth., *25:* 58.

———— 1941b The nature of experimental degeneration of the retina. Brit. J. Ophth., *25:* 62.

STUDNIČKA, F. K. 1898 Untersuchungen über den Bau der Sehnerven der Wirbeltiere. Yena. Zeits. f. Naturwiss. Bd., *31:* 1.

VERHOEFF, F. H. 1931 Microscopic observations on a case of retinitis pigmentosa. Arch. Ophth., *5:* 392.

WALD, G., AND P. K. BROWN 1951 Role of sulfhydryl groups in the bleaching and synthesis of rhodopsin. Fed. Proc., *10:* 266.

WALLS, G. L. 1942 "The Vertebrate Eye." Crankbrook Inst. of Science, Bulletin No. 19.

PLATE 1

EXPLANATION OF FIGURES

Rabbit's retina three weeks after iodoacetate administration.

A Midperipheral segment of retina which has become detached during the histological preparation. (In this and *all other pictures* vitreal side of retina is upward.) Compared with normal retina (right side) one notes that all visual cells have disappeared, except for one pyknotic nucleus. The ganglion cells and the inner nuclear layer are preserved. The outer limiting membrane (arrow) has become detached from an intact pigment epithelium (lower portion of the picture). Hematoxylin-eosin × 330.

B Upper portion of section shows retina, lower portion choriodal structures, the outer elastic sheet of the glass-membrane (arrow) separating them. Visual cells and inner nuclear layer are preserved. Some Müller cells have invaded the space between inner nuclear layer and choriod. Hematoxylin-eosin × 330.

C Anterior segment of retina, broken at ora serrata (left corner). Outer nuclear layer decreases in width with increasing distance from ora serrata. Heidenhain phloxine × 100.

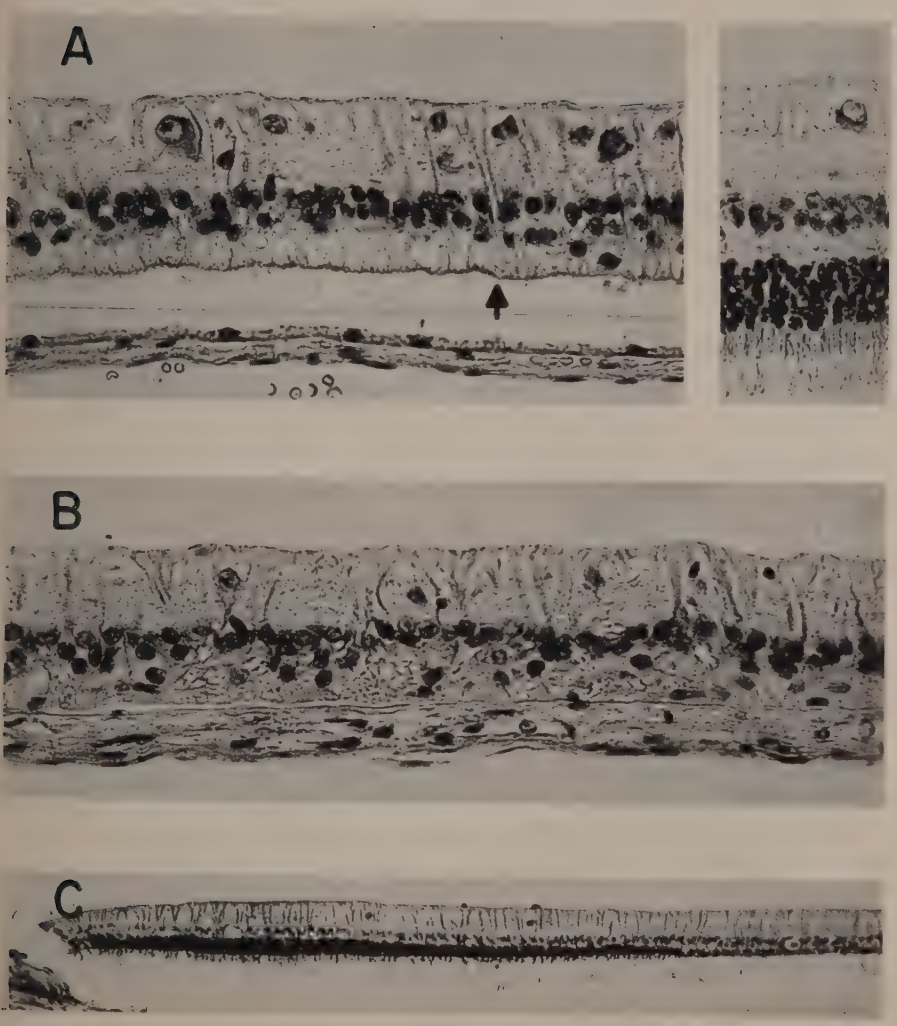
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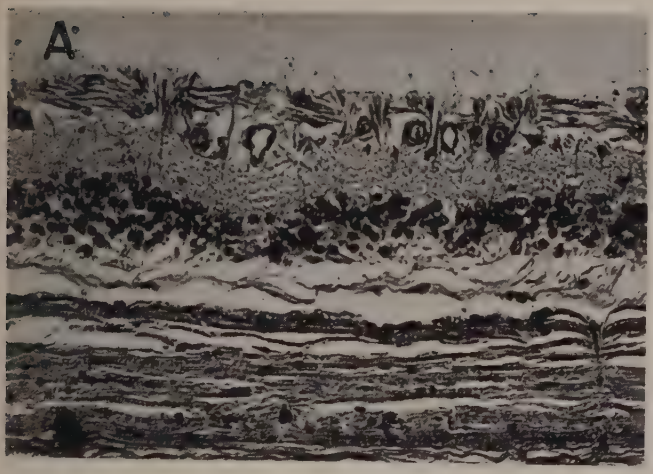
PLATE 2

EXPLANATION OF FIGURES

Cat's retina three weeks after two injections of iodoacetate.

A Midperipheral segment of retina and tapetum cellulosum. Visual cells have disappeared. Pigment cells are partially preserved. Inner nuclear layer is distorted. Compare with normal retina, corresponding segment (right side of picture). Hematoxylin-eosin × 250.

B Another segment of same retina where pigment cells contain normally pigment (no tapetum). Pigment has accumulated within retinal layers. Hematoxylin-eosin × 250.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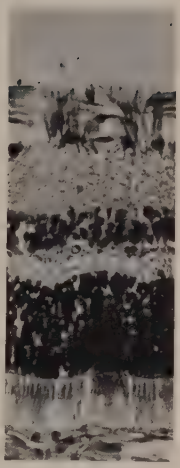

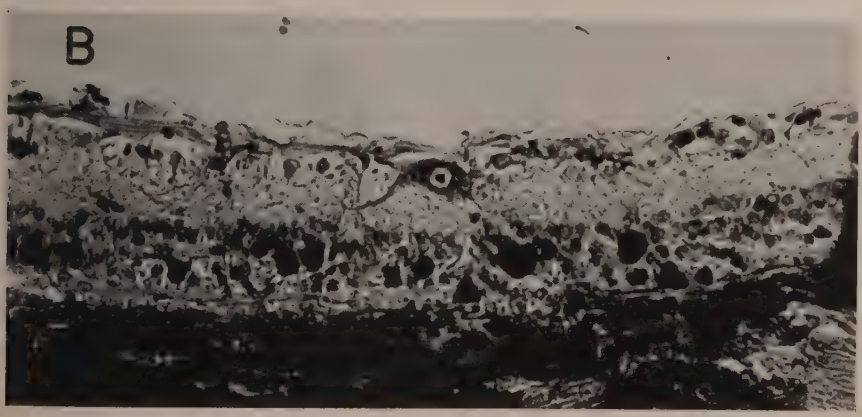
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PLATE 3

EXPLANATION OF FIGURES

Cone cells from Macaca mulatta; 5 weeks after last and 7 weeks after first injection of iodoacetate. Heidenhain-phloxine × 620.

A Central region of fovea centralis; visual cell layer. Cone cells have normal appearance.

B Parafovea about 1 mm distance from central region of fovea; visual cell layer. Cone cells are abnormal (see description in text). Outer nuclear layer is decreased in width because of the disappearance of rod cells.

C Parafovea, about 2 mm distance from center of fovea; visual cell layer. Cone cell change has continuously increased in severity with greater distance from fovea. Beneath the cone cell layer are some pyknotic nuclei.

D About 4 mm distance from center of fovea. Inner segment of cone cells has almost completely vanished.

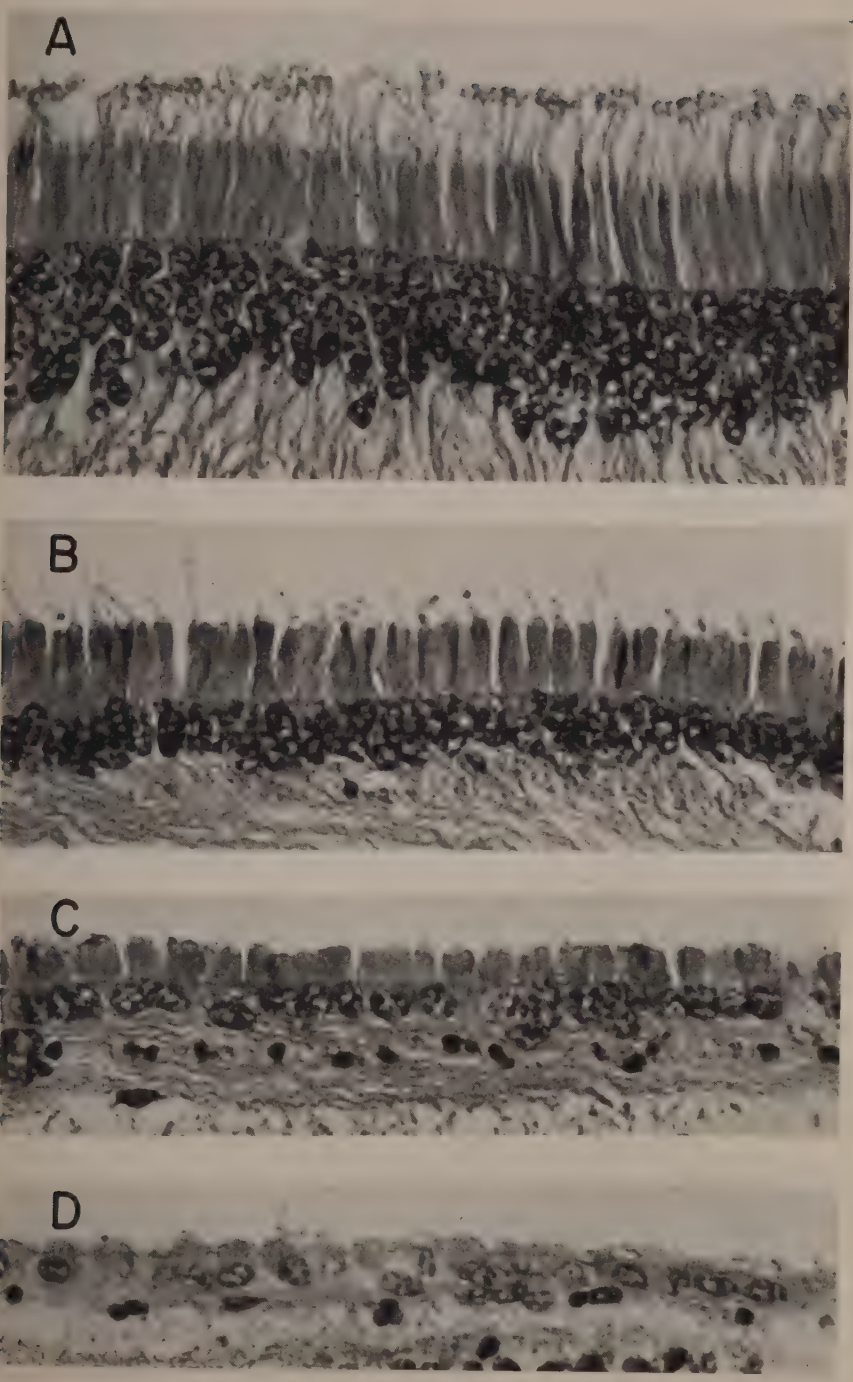
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PLATE 4

EXPLANATION OF FIGURES

A Macaca mulatta (continued from plate 3, D). Retinal segment about 2 mm to the nasal side of optic disc; visual cell layer and inner nuclear layer. Cone cells form a "simple" epithelial layer. They contain remnants of the ellipsoid body. All rod cells have disappeared. Inner nuclear layer is of normal thickness (4 rows of nuclei). Heidenhain-phloxine × 620.

B Macaca mulatta (continued from plate 3). Periphery of retina; visual cell layer and inner nuclear layer. Rod cells are absent. Cone cell remnants (nucleus plus ellipsoid body) interrupt the outer limiting membrane. Inner nuclear layer has normal width. Heidenhain-phloxine × 620.

C Macaca mulatta (continued from plate 3). Periphery of retina about 5 mm away from ora serrata. Cone cell nuclei and several rod cell nuclei are preserved. In center of picture are 6 deformed inner rod segments. Heidenhain-phloxine × 620.

D Cat's retina; visual cell layer. Six days after one injection of iodoacetate. Arrow — outer limiting membrane. Outer and inner segments have disappeared, except for some remnants of inner segments (center of picture). Most outer nuclei are pyknotic. In the outermost row, nuclei with a reticular, cone-like organization of chromatin have survived. Heidenhain-phloxine × 1000.

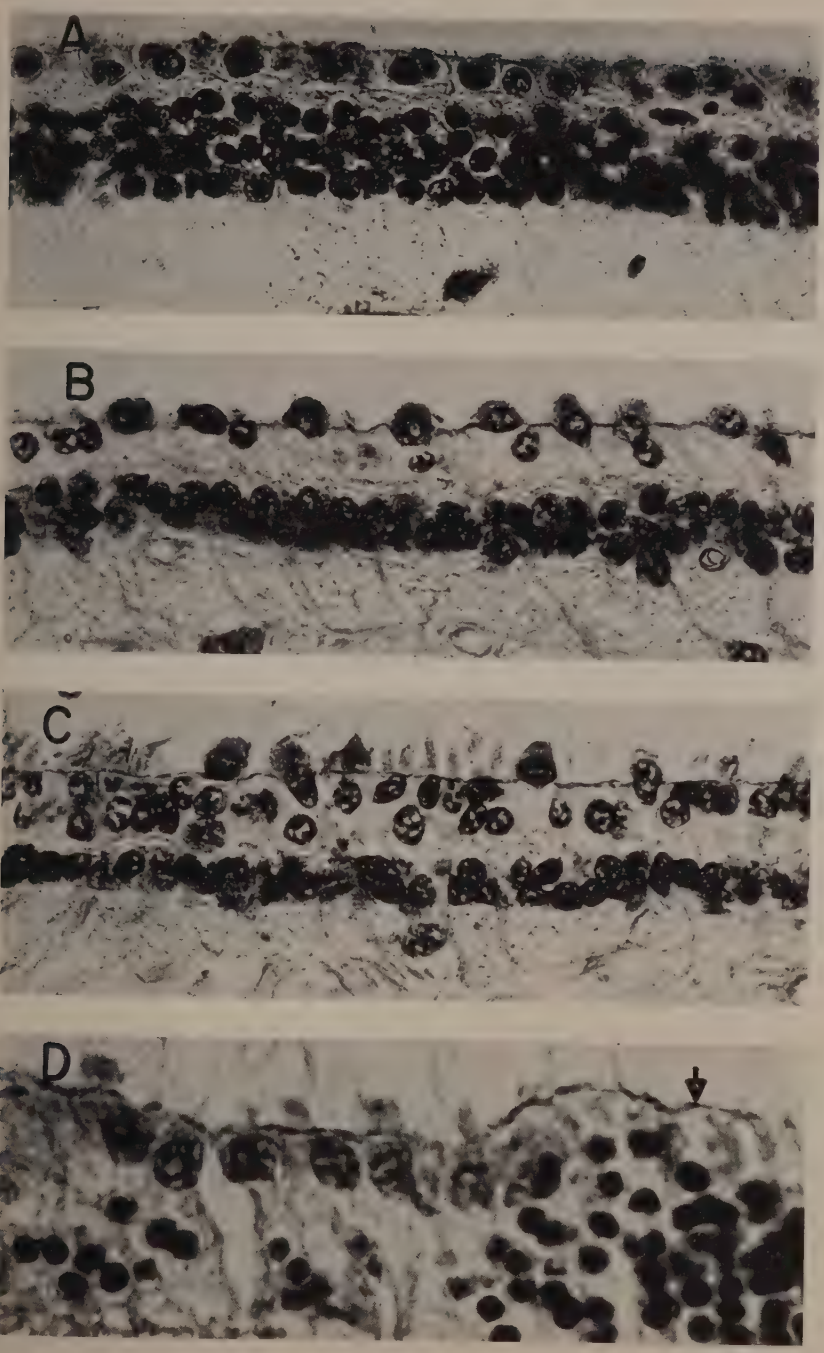
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PLATE 5

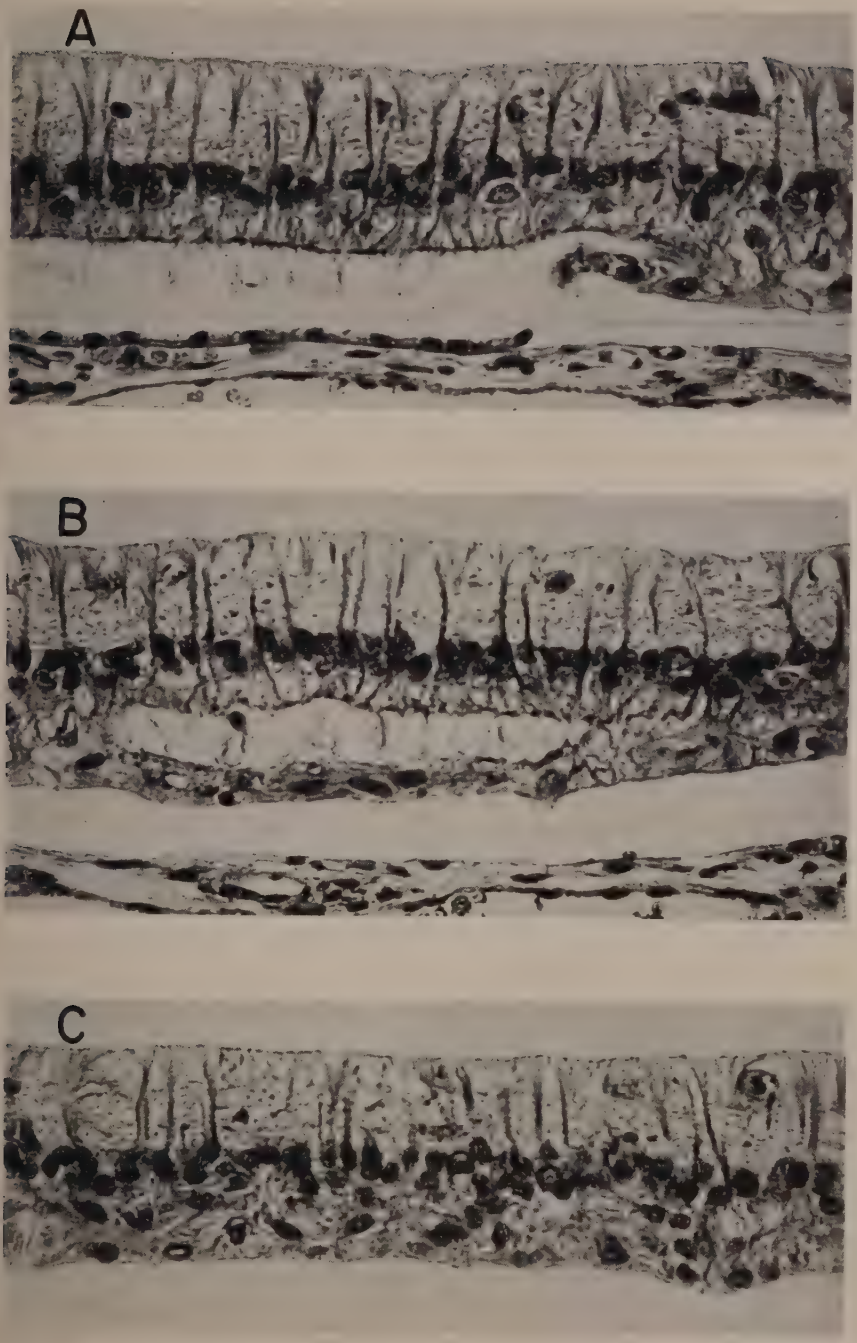
EXPLANATION OF FIGURES

Rabbit's retina; three weeks after iodoacetate administration. Three millimeters distance from ora serrata. Heidenhain-phloxine × 400.

A Visual cells have disappeared. Retina has become detached. At the left, glia fibers extend into the open space between outer limiting membrane and pigment epithelium (which is separately photographed). At right lower corner pigment epithelium disappears abruptly and a tissue bridge exists between the end of the preserved epithelium and the outer plexiform layer (broken during fixation), the outer limiting membrane is disrupted.

B Continues A (pictures overlap). Outer limiting membrane reappears for a short distance. Pigment epithelium remains absent. A band of Müller cells crosses through the space between the choriod (below, separately photographed) and the preserved outer limiting membrane. At right corner outer limiting membrane disappears again and the newly grown Müller cells form the border of the retina.

C Continues B. Beneath the inner nuclear layer newly grown Müller cells tend to take a horizontal position. Outer limiting membrane has disappeared. Müller cells form the border of retina which has become detached from choriod as in B; see also plate 1, B.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STUDIES ON PHOSPHORUS METABOLISM IN SEA URCHIN EMBRYOS[1]

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The rates of incorporation of P^{32} (as inorganic phosphate) from sea water into the several phosphate fractions of sea urchin embryos during early development was studied previously (Villee et al., '49). These experiments showed that the desoxypentose nucleic acid (DNA) synthesized during development from fertilized egg to swimming gastrula did not come from the pentose nucleic acid (PNA) of the unfertilized egg (Brachet, '33, '37) and rendered unlikely the hypothesis that it is formed from a PNA precursor. Certain drugs known to retard cleavage were tested for their effects on phosphate metabolism. Dinitrophenol (5×10^{-5} M) and uranyl nitrate (10^{-4} M) reduced the uptake of P^{32} into the acid soluble phosphorus fraction of the cells but had little additional effect on nucleic acid metabolism. Malononitrile (10^{-2} M) and tris (β-chloroethyl) amine (10^{-4} M) had only a slight effect on phosphate uptake but had more marked effects on nucleic acid metabolism; malonitrile had a greater inhibitory effect on PNA synthesis and the nitrogen mustard had a greater inhibitory effect on DNA synthesis. A similar inhibition of DNA but not of PNA synthesis in developing frog embryos was reported for another nitrogen mustard, methyl bis (β-chloroethyl) amine by Bodenstein and Kondietzer ('48).

The alkaloids pilocarpine, atropine and eserine, which affect primarily the neuro-effectors of vertebrates, had been

[1] Supported in part by a grant from the Charles A. King and Marjorie King Fund.

shown to have effects on cell division (Mathews, '02; Sollman, '06) and on cellular respiration (Deutsch and Raper, '36, '38). Mathews ('02) found that pilocarpine speeded and atropine retarded sea urchin development. He suggested that cellular oxidations were increased by pilocarpine and inhibited by atropine. Sollman ('06), using *Fundulus* eggs, found that pilocarpine retarded development and that atropine was without effect. Pilocarpine, acetyl choline, and eserine were found to increase the respiration, *in vitro,* of slices of cat submaxillary gland (Deutsch and Raper, '36, '38). To gain further information about the correlation between cleavage and nucleic acid synthesis, developing sea urchin eggs were exposed to P^{32} and to various concentrations of the drugs reputed to have effects on the rate of development.

MATERIALS AND METHODS

Eggs were collected by injecting several female sea urchins *(Arbacia punctulata)* with 0.53 M KCl. The eggs were washed in sea water and examined under the microscope to insure that they were neither cytolyzed nor fertilized. The experiments were carried out in finger bowls, in each of which was placed 25 ml of egg suspension, 5 μC of P^{32}, a solution of the drug being tested, and sea water to a total volume of 100 ml. The drug solutions were made fresh each time. The amount of phosphorus added as P^{32} was negligible in comparison to the amount already present in sea water. The eggs were fertilized by adding three drops of dilute sperm suspension and development was allowed to proceed at room temperature (23°C.) for 7½ hours. With each experiment, one bowl, to which no drug was added, served as a control. Periodically during the experiment and at the end, eggs were examined under the microscope and the degree of development was recorded. At the end of the experiment, the embryos were removed from the finger bowls, centrifuged lightly to remove the P^{32}-containing sea water, rinsed in fresh sea water, and centrifuged again. Approximately 0.6 to 0.8 gm (wet weight) of embryos were recovered from each bowl. Twenty milli-

liters of ice-cold 7% trichloracetic acid were added to the embryos and they were homogenized. The resulting suspension was stirred mechanically for 10 minutes and then fractionated into acid-soluble phosphorus, lipid phosphorus, and acid-insoluble phosphorus, using the Schmidt-Thannhauser ('45) procedure, and the acid insoluble phosphorus fraction was further subdivided into desoxypentosenucleic acid P, pentose nucleic acid P, and phosphoprotein P as described in detail previously (Villee et al., '49). Aliquots of all fractions were analyzed for total phosphorus by the method of Fiske and Subbarow ('25) and for P^{32} using the dip counter described by Solomon and Estes ('48). The fractionation scheme permits one to make independent, direct estimations of the DNA phosphorus, PNA phosphorus, and phosphoprotein phosphorus, as well as of total acid insoluble phosphorus and of PNA plus phosphoprotein phosphorus. The sums of the individual P^{32} analyses and the P^{32} analysis of the total acid insoluble phosphorus fraction agreed within 4% and the sums of the individual Fiske and Subbarow determinations corresponded with the total acid insoluble phosphorus within 10%.

RESULTS

1. General. All the drugs tested showed a marked (50–200%) acceleration in phosphorus metabolism at low concentrations, 6 mg per 100 ml, and an inhibition of growth and phosphorus metabolism at higher concentrations, 60, 100, or 500 mg per 100 ml. Only acetyl choline produced a significant acceleration of cleavage and growth rate at low concentration. Similar curves of excitation at low concentrations and inhibition at higher ones are usually observed in studies of the effects of these drugs on vertebrate tissues. The order of effectiveness of the drugs in producing inhibition of cleavage and phosphorus metabolism was eserine>pilocarpine>atropine>acetylcholine. The explanation for the fact that Mathews ('02) found that pilocarpine speeded development and Sollman ('06) found that it inhibited development proba-

bly lies in the different concentrations used. Mathews used solutions of about 1:100,000 and Sollman used solutions of 1:10,000. The drugs inhibited growth at low concentration by retarding development, not by killing the organisms. This was shown by allowing the remaining embryos to develop *ad libitum* after a portion had been removed and used for chemical and radiochemical analyses. These embryos lived up to 14 days and there was no differential death rate between experimental and control animals. The embryos developing in higher concentrations, 60 or 100 mg eserine or pilocarpine per 100 ml sea water, died after 28 hours of incubation and, although they became gastrulas, they never exhibited swimming motion.

2. Eserine. In each experiment, the control embryos were fractionated simultaneously with those to which varying amounts of the drug had been added. From the P^{32} analyses and the phosphorus analyses, the specific activity, i.e., the counts per minute per milligram of phosphorus, was calculated for each fraction. The specific activity of a given fraction from embryos treated with a given dose of drug was then divided by the specific activity of the comparable fraction from the control embryos raised simultaneously and taken from the same batch of eggs and sperm. The resulting figure, the relative specific activity, gives a measure of the effect of the drug on the incorporation of phosphorus into that particular fraction. The effect of the varying concentrations of eserine on cell division and phosphorus metabolism is shown in table 1. The "total acid soluble P" fraction includes inorganic phosphorus, and small organic phosphates such as hexose and triose phosphate, adenosine triphosphate, and so on. The incorporation of P^{32} into this fraction is a measure of its uptake into the cell and hence of its availability for further synthetic reactions. Evidence from a variety of sources, Kamen and Spiegelman ('48), Sacks ('48), Villee et al. ('49), indicates that inorganic phosphate from the medium is taken into cells by an enzyme-controlled reaction. The incorporation of P^{32} into this fraction is stimulated by

TABLE 1

Effect of eserine on development and phosphorus metabolism in Arbacia embryos

ESERINE CONCENTRATION	STAGE ATTAINED 7½ HRS. AFTER FERTILIZATION	RELATIVE SPECIFIC ACTIVITY, I.E., counts/min./mg P of treated embryos / counts/min./mg P of control embryos				
		Acid soluble P	Phospho-lipid P	DNA P	PNA P	Phospho-protein P
Control	Late blastula	1.00	1.00	1.00	1.00	1.00
1.85×10^{-4} M	Late blastula	1.89	1.02	1.17	1.62	1.35
3.08×10^{-4} M	Mostly blastulas; a few 2, 4, 8, and 16 cell	1.82	1.01	0.46	1.44	1.15
7.72×10^{-4} M	Mostly blastulas; some 8 and 16; a few 2 and 4	1.59	0.91	0.48	1.33	1.12
1.85×10^{-3} M	Few blastulas; few 16 and 32; mostly 4 and 8, few 2	0.96	0.82	0.25	0.94	1.09
3.08×10^{-3} M	Mostly 1 cell; few 2, 4 and 8; none higher	0.54	0.83	0.16	0.93	0.81

low concentrations, and inhibited by higher concentrations, of eserine. At an intermediate concentration, 1.85×10^{-3} M, the incorporation is at the control level. This suggests that eserine affects the enzyme system responsible for the uptake of phosphorus by the developing egg, accelerating it at low concentrations and inhibiting it at higher ones. The decrease in the incorporation of P^{32} into the total acid insoluble P fraction, the lipid phosphorus, the PNA phosphorus and the phosphoprotein phosphorus at higher eserine levels could be due simply to the decreased amount available because of the inhibition of this phosphorus uptake system. However, the incorporation of P^{32} into DNA phosphorus is inhibited much more than the incorporation into other fractions: the inhibition begins at lower eserine concentration (3.08×10^{-4} M), and at each higher concentration there is much greater inhibition, i.e., the relative specific activity is lower, in the incorporation of P^{32} into the DNA phosphorus fraction than into any other fraction. This suggests that eserine has, in addition to its effect on the enzyme responsible for the uptake of phosphorus by the cell, an effect on one or more of the enzymes involved in the synthesis of desoxypentose nucleic acid. This inhibition of cleavage and development by eserine is paralleled only by the inhibition of the incorporation of P^{32} into DNA P and not by the incorporation of P^{32} into any of the other fractions. The work of Boivin, Vendrely and Vendrely ('48) and of Mirsky and Ris ('48) indicates that adult animal cells are characterized by a relatively constant amount of DNA per cell. This may also be true of rapidly dividing embryonic cells, and DNA synthesis and cell division may be connected in some causal fashion. The incorporation of P^{32} into PNA and phosphoprotein, in contrast, does not appear to be decreased except by the decrease in the availability of P^{32} because of the effect of eserine on the uptake of phosphorus by the cell.

3. Pilocarpine. The relative specific activities of the several fractions isolated from embryos grown in sea water con-

TABLE 2

Effect of pilocarpine on development and phosphorus metabolism in Arbacia embryos

PILOCARPINE CONCENTRATION	STAGE ATTAINED 7½ HRS. AFTER FERTILIZATION	RELATIVE SPECIFIC ACTIVITY, I.E., counts/min./mg P of treated embryos / counts/min./mg P of control embryos				
		Acid soluble P	Phospho-lipid P	DNA P	PNA P	Phospho-protein P
Control	Swimming gastrulas	1.00	1.00	1.00	1.00	1.00
2.21×10^{-4} M	25% gastrulas; 75% blastulas	0.93	0.56	0.94	0.81	0.96
3.69×10^{-4} M	20% gastrulas; 70% blastulas; 10% 8 and 16 cell	0.81	0.67	0.94	0.92	1.19
9.22×10^{-4} M	10% gastrulas; 60% blastulas; 30% 2, 4, 8 and 16 cell	0.87	1.00	1.01	1.46	1.14
2.21×10^{-3} M	5% gastrulas; 60% blastulas; 35% 2, 4, 8 and 16 cell	0.49	0.73	0.50	0.94	0.83
3.69×10^{-3} M	all 1 cell stage; fertilization membranes visible	0.26	0.56	0.47	1.13	0.43

taining varying amounts of pilocarpine are given in table 2. It can be seen that pilocarpine is almost as effective as eserine in inhibiting cleavage and even more effective in decreasing the uptake of phosphorus into the acid soluble phosphorus fraction. It does not have as marked an additional effect on the synthesis of desoxypentose nucleic acid as eserine does, for although the incorporation of P^{32} into DNA is decreased by 2.21×10^{-3} M or 3.69×10^{-3} M pilocarpine, the decrease is less than that found with eserine and less than the inhibition of P^{32} incorporation into the acid soluble phosphorus fraction. It might be argued that all of the decrease of P^{32} incorporation into DNA is due to the decreased amount present in the cell; however, the incorporation of P^{32} into PNA is unimpaired by either 2.21×10^{-3} M or 3.69×10^{-3} M pilocarpine, as it was with similar concentrations of eserine. Thus, with pilocarpine as with eserine there seems to be, in addition to an inhibition of P^{32} uptake by the cell, an inhibition of DNA synthesis correlated with the inhibition of cleavage.

4. Atropine. In contrast to eserine and pilocarpine, atropine has no effect on cleavage or phosphorus metabolism at levels up to 2.95×10^{-3} M. It does, however, have inhibiting effects at a concentration of 1.48×10^{-2} M. At this level, the incorporation of P^{32} into DNA is inhibited more than the incorporation of P^{32} into other fractions (table 3).

5. Acetyl choline. Acetyl choline had the most marked effect of stimulating cleavage at low concentrations (2.65×10^{-4} M) of any substance tested. The incorporation of P^{32} into all fractions was enhanced by this concentration of acetyl choline. Table 4 shows that, although neither 1.85×10^{-4} M eserine nor 2.65×10^{-4} M acetyl choline decreased DNA synthesis, 1.85×10^{-4} M eserine plus 2.65×10^{-4} M acetyl choline had as much inhibitory effect as 3.08×10^{-4} M eserine (table 1). This experiment was repeated with identical results: the relative specific activity of the DNA phosphorus from 1.85×10^{-4} M eserine was 1.04, from the 2.65×10^{-4} M

TABLE 3

Effect of atropine on development and phosphorus metabolism in Arbacia embryos

ATROPINE CONCENTRATION	STAGE ATTAINED 7½ HRS. AFTER FERTILIZATION	RELATIVE SPECIFIC ACTIVITY, I.E., counts/min./mg P of treated embryos / counts/min./mg P of control embryos				
		Acid soluble P	Phospho-lipid P	DNA P	PNA P	Phospho-protein P
Control	Late blastula	1.00	1.00	1.00	1.00	1.00
2.95×10^{-4} M	Late blastula	1.97	1.02	1.99	1.05	1.04
7.40×10^{-4} M	Late blastula	1.10	1.40	1.15	1.02	1.43
2.95×10^{-3} M	Blastula	2.05	0.78	1.29	0.93	1.01
1.48×10^{-2} M	2 and 4 cell; few 8 cell; none higher	0.30	0.38	0.10	0.18	0.18

TABLE 4

Effect of acetyl choline and acetyl choline plus eserine on development and phosphorus metabolism in Arbacia embryos

CONCENTRATION	STAGE ATTAINED 7½ HRS. AFTER FERTILIZATION	RELATIVE SPECIFIC ACTIVITY, I.E., counts/min./mg P of treated embryos / counts/min./mg P of control embryos				
		Acid soluble P	Phospho-lipid P	DNA P	PNA P	Phospho-protein P
Control	75% blastulas; 25% gastrulas	1.00	1.00	1.00	1.00	1.00
2.65×10^{-4} M Ach	all swimming gastrulas	1.17	1.55	1.19	1.10	1.21
2.65×10^{-3} M Ach	50% blastulas; 50% gastrulas	0.84	0.51	0.84	0.59	1.33
1.85×10^{-4} M E	75% blastulas; 25% gastrulas	1.70	1.13	1.09	1.05	1.00
1.85×10^{-4} M E plus 2.65×10^{-4} M Ach	50% blastulas; 50% gastrulas	1.15	1.41	0.55	0.99	1.20

acetyl choline was 1.11, and from 1.85×10^{-4} M eserine plus 2.65×10^{-4} M acetyl choline was 0.53. When the acetyl choline concentration was raised to 2.65×10^{-3} M there was a slight inhibition of P^{32} incorporation into all fractions, with stronger inhibitions of phospholipid and PNA phosphorus than of acid soluble and DNA phosphorus.

6. Temperature. Previous experiments (Villee et al., '49) had shown that low temperature markedly reduced the uptake of P^{32} by fertilized *Arbacia* eggs and the conclusion was drawn that phosphorus enters the cell by an enzyme-controlled metabolic process rather than by simple diffusion. To investigate this phenomenon further, eggs were fertilized in bowls of sea water containing 3 to 5 μC of P^{32} and incubated $7\frac{1}{2}$ hours at controlled temperatures ranging from —12°C. to 28°C. After incubation, they were homogenized and fractionated as before and the specific activities of the several fractions were calculated. Relative specific activities were then calculated as fractions of the specific activities at the highest temperature used, 28°C., and are recorded in table 5. Low temperatures, like high concentrations of the drugs used in other experiments, decrease the rate of development; no cleavage divisions occurred at —12°C. or at 0°C. However, the effects of low temperature on phosphorus metabolism differ from those produced by drugs, for the incorporation of P^{32} into all fractions is inhibited, more or less equally, by low temperature, whereas the incorporation of P^{32} into DNA is differentially affected by most of the drugs used.

The marked decrease in the incorporation of P^{32} into the acid soluble phosphorus fraction at low temperatures confirms and extends the previous observations (Villee et al., '49) and is in accord with the hypothesis that phosphorus enters the cell by a metabolic, enzyme-controlled process rather than by simple diffusion (Kamen and Spiegelman, '48; Sacks, '48).

TABLE 5

Effect of temperature on development and phosphorus metabolism in Arbacia embryos

TEMP.	NO. OF EXPTS.	STAGE ATTAINED 7½ HRS. AFTER FERTILIZATION	RELATIVE SPECIFIC ACTIVITY, I.E., counts/min./mg P of embryos raised at given temp. / counts/min./mg P of embryos raised at 28°C.				
			Acid soluble P	Phospho-lipid P	DNA P	PNA P	Phospho-protein P
— 12°C.	3	All one cell; fertilization membranes visible	.03	.13	.03	.06	.08
0°C.	1	All one cell; fertilization membranes visible	.09	.52	.10	.22	.20
5°C.	2	92% one cell; 8% two cell; none higher	.11	.24	.16	.11	.74
10°C.	3	5% two cell; 60% four cell; 30% eight cell; 5% 16 cell	.47	.20	.05	.24	.48
15°C.	1	32 and 64 cell stages	.50	.31	.16	.51	.87
18°C.	3	All blastulas	.59	.30	.31	.38	.87
25°C.	2	50% blastulas; 50% swimming gastrulas	.92	.90	.71	.94	
28°C.	3	10% blastulas; 90% swimming gastrulas	1.00	1.00	1.00	1.00	1.00

DISCUSSION

The effects of these drugs on development appear to be mediated by mechanisms that are different from their usual ones in neuroeffectors. The concentrations used in these studies were much higher than those commonly used in pharmacological investigations and it is possible that the effects observed were due to some general chemical property common to the alkaloids rather than to the specific pharmacological properties of the individual drugs which become apparent at lower concentrations. Acetyl choline and pilocarpine produce parasympathetic stimulation, eserine (physostigmine) inhibits cholinesterase and thus preserves acetyl choline, and atropine is an antagonist of acetyl choline and pilocarpine. Eserine is believed to act as a competitive inhibitor of cholinesterase. Since it is unlikely that any cholinesterase is present in cleaving sea urchin eggs, or, if there is, that cholinesterase has anything to do with either phosphorus uptake or desoxypentose nucleic acid synthesis, it appears likely that these drugs are affecting enzymes other than cholinesterase. In their effects on vertebrate tissues they frequently cause accelerations at low concentrations and inhibitions at higher concentrations; similar phenomena were observed in their effects on phosphorus metabolism.

Whatever the mode of action of these drugs on sea urchin eggs may be, these experiments have shown that when growth is inhibited, the incorporation of P^{32} into DNA is also inhibited. In contrast, it is possible to have complete inhibition of growth, so that all the eggs remain in the one-cell stage, and have the incorporation of P^{32} into PNA proceed at the normal rate. There is a steadily mounting body of evidence (Boivin, Vendrely and Vendrely, '48; Mirsky and Ris, '48) that there is a constant amount of DNA per cell in any given species, as one would expect since DNA is largely in the chromosomes and the number of chromosomes per cell is constant for any species. If we may take the rate of incorporation of P^{32} into DNA as a measure of the rate of synthesis of DNA, our data indicate that when cell division is prevented, DNA synthe-

sis is inhibited, as one would expect if there were a constant amount of DNA per cell. It is impossible to determine from these experiments whether the drug primarily inhibits some enzyme system responsible for DNA synthesis and then cell division is prevented secondarily due to the lack of DNA, or whether the drug primarily affects cell division, as colchicine (another alkaloid) does, and the effect on DNA synthesis is secondary. Whether cell division is inhibited by a drug or by low temperature, the incorporation of P^{32} into DNA is inhibited in a parallel fashion.

SUMMARY

1. Eserine, pilocarpine, atropine and acetyl choline, when present in the sea water in which sea urchin eggs are developing, accelerate phosphorus metabolism at low concentrations and inhibit it at higher concentrations. Eserine, and, to a lesser extent, pilocarpine and atropine, inhibit the incorporation of P^{32} into desoxypentose nucleic acid but not the incorporation of P^{32} into pentose nucleic acid; they inhibit cleavage in a parallel fashion.

2. When developing sea urchin eggs are kept at low temperatures, cleavage is inhibited and the incorporation of P^{32} into all phosphorus fractions, acid soluble, phospholipid, desoxypentose nucleic acid, pentose nucleic acid, and phosphoprotein, is inhibited more or less equally. If phosphorus entered the cell by simple diffusion, its rate of entrance (incorporation into acid soluble phosphorus) would be proportional to the absolute temperature; these experiments show that the mechanism responsible for phosphorus uptake has a Q_{10} of about 2 over the range $-12°$C. to 28°C. and thus is presumed to be an enzyme-controlled process.

LITERATURE CITED

BODENSTEIN, O., AND A. A. KONDIETZER 1948 The effect of nitrogen mustard on nucleic acids during embryonic amphibian development. J. Exp. Zool., *107:* 109–121.

BOIVIN, A., R. VENDRELY AND C. VENDRELY 1948 L'acide désoxyribosnucléique du noyau cellulaire, dépositaire des caractères hérèditaires; arguments d'ordre analytique. C. R. Acad. Sci., *226:* 1061-1063.

BRACHET, J. 1933 Recherches sur le synthèse de l'acide thymonucléique pendant le développement de l'oeuf d'oursin. Arch. Biol., Paris, *44:* 519–576.

———— 1937 Remarques sur le formation de l'acide thymonucleique pendant le développement des oeufs a synthèse partielle. Arch. Biol., Paris, *48:* 529–540.

DEUTSCH, W., AND H. S. RAPER 1936 Respiration and functional activity. J. Physiol., *87:* 275–286.

———— 1938 The respiration and metabolism of submaxillary gland tissue of the cat. J. Physiol., *92:* 439–458.

FISKE, C. H., AND Y. SUBBAROW 1925 The colorimetric determination of phosphorus. J. Biol. Chem., *66:* 375–400.

KAMEN, M. D., AND S. SPIEGELMAN 1948 Studies on the phosphate metabolism of some unicellular organisms. Cold Spring Harbor Symp. Quant. Biol., *13:* 151–163.

MATHEWS, A. P. 1902 The action of pilocarpine and atropine on the embryos of the starfish and the sea urchin. Am. J. Physiol., *6:* 207–218.

MIRSKY, A. E., AND H. RIS 1949 Variable and constant components of chromosomes. Nature, *163:* 666–667.

SACKS, J. 1948 Mechanism of phosphate transport across cell membranes. Cold Spring Harbor Symp. Quant. Biol., *13:* 180–184.

SCHMIDT, G., AND S. J. THANNHAUSER 1945 A method for the determination of desoxyribonucleic acid, ribonucleic acid, and phosphoproteins in animal tissues. J. Biol. Chem., *161:* 83–89.

SOLLMAN, T. 1906 The effects of a series of poisons on adult and embryonic *Funduli.* Am. J. Physiol., *16:* 1–46.

SOLOMON, A. K., AND H. D. ESTES 1948 The measurement of radioactivity in solution. Rev. Sci. Instr., *19:* 47–50.

VILLEE, C. A., M. LOWENS, M. GORDON, E. LEONARD AND A. RICH 1949 The incorporation of P^{32} into the nucleoprotein and phosphoproteins of the developing sea urchin embryo. J. Cell. and Comp. Physiol., *33:* 93–112.

SOME EFFECTS OF INORGANIC IONS ON THE ACTIVE TRANSPORT OF PHENOL RED BY ISOLATED KIDNEY TUBULES OF THE FLOUNDER [1,2]

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THREE FIGURES

Forster ('48) and Forster and Taggart ('50) demonstrated that the tubules of the flounder kidney can be used in vitro as an elegant preparation for the study of active transport. The ease with which these tubules separate when shaken in modified Ringer's solution makes possible direct microscopic observation of the accumulation of phenol red inside the lumen. Cross and Taggart ('50) developed a procedure for studying metabolic aspects of tubular excretion in mammalian kidney slices, which depends on measurement of the material taken up by the slice from the surrounding medium. This method, however, does not permit direct observation of the excretory process. The present communication describes experiments involving an adaptation of Forster's technique to permit semi-quantitative estimation of the transport of phenol red, without sacrifice of the ability to observe excretion directly.

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MATERIALS AND METHODS

Kidney tissue of freshly caught flounder was removed immediately after capture of the fish. The tubules were carefully teased apart and approximately 5–10 mg quantities placed in small petri dishes containing 5.0 cm^3 of Ringer's solution and phenol red, actively aerated with water-saturated O_2, as described by Foster and Taggart ('50). A series of 7 or 14 vessels was employed in each experiment so that test and control observations could be performed simultaneously on tissue from the same fish. During aeration the dishes were kept in a water bath the temperature of which was maintained by manual adjustment. Except where otherwise noted, the experimental temperature was 28° ± 3°C., at which point the ability of the tubule to transport phenol red is maximal.[5]

In order to measure the uptake of dye by the kidney, a color-comparison block was constructed permitting the intensity of the dye concentrated within the tubule to be matched with a graduated series of standards, while viewed under the microscope. The comparator consisted of a block of lucite $8 \times 2 \times 0.25$ cm in which 8 holes of 0.4 cm diameter were drilled. Another flat lucite plate was sealed to the bottom of the first with Duco cement. A series of 7 standard dye solutions was freshly prepared every other day with the following concentrations of phenol red: 0.005, 0.010, 0.020, 0.040, 0.080, 0.16, and 0.32 mg/cm^3. In addition, the standard solutions contained 0.12 M $NaHCO_3$ to control pH and 1.6% bacto agar to prevent the liquid from leaking out of the interstices in the comparator. Test showed that the presence of the agar did not significantly affect the color value of the dye standards. In preparing the comparator the standard solutions were warmed to melt the agar, and then pipetted into the appropriate holes in the lucite block. After the agar had jelled, the excess rounded droplet on top of each hole was carefully removed, leaving the top of the dye column exactly flush with the top of the plate. A strip of scotch tape was then laid over all the holes and the comparator mounted in position on the microscope stage, so that its top face was almost in contact with the glass dish containing the tubule specimens.

In making readings, each petri dish was removed temporarily from the water bath and placed on top of two supports on the microscope

[5] At higher temperatures loss of the capacity to transport phenol red is irreversible in contrast to the reversible depression of activity occurring when the suspension is chilled. It is noteworthy that in these experiments discharge of the accumulated dye from a tubule preparation occurred within a few minutes after cooling to ice-bath temperatures; whereas Forster and Taggart ('50) reported that several hours were required for low temperature inhibition to take effect.

stage, adjusted so that just enough room was allowed for the comparator block to slide across the field of view underneath the dish. A blue filter in front of the microscope lamp absorbed enough of the red component of the illumination, so that when viewed under the microscope, the solution bathing the tissue (which usually contained 0.01 mg/cm^3 of phenol red and had a depth of 2 mm) appeared practically devoid of red color. Thus, it was unnecessary to control more than approximately the depth of the outer solution, since small variations in this level did not appreciably affect the color-matching process. When much higher concentrations of the dye were employed in the external solution, the excess liquid was decanted just before the microscopic examination.

With the microscope (50 ×–100 × magnification) focused on a tubule, the comparator was slid along its track directly in the line of focus until an approximate match of the color in the tubule with one of the standard colors was secured. The final comparison was made with part of the tubule in question lying across the color standard, and part extending beyond. If the color intensity in a given tubule was intermediate between that of two consecutive standards, the midpoint dye concentration was assigned it. The depth of the dye solution in the comparator block was 0.25 cm. The dimensions of the kidney tubules in the flounder were measured with a calibrated micrometer eye-piece and found to be sufficiently constant in different specimens so that the following average values could be used for all the experiments, well within the limits of precision of the over-all procedure: lumen diameter, 0.0019 cm; total tubule diameter, 0.0053 cm. Thus, the concentration of dye in either the lumen or the cells of a tubule is given by the expression:

$$\frac{\text{Concentration in standard} \times \text{depth of standard}}{\text{depth of structure}}$$

Determinations were carried out on the tubules lying lengthwise in the field of view. Hence, in estimating the dye concentration in a lumen, 0.0019 cm was used for the denominator, while for dye accumulation in the single-cell layer constituting the tubule wall, the value 0.0036 cm was calculated from geometrical considerations as the average optical depth of the structure.

Evaluation of the dye concentration in the lumen or in the cells of any given tubule was found to be an unequivocal process, and different observers consistently agreed on such readings of a given tubule. It was more difficult to assign a value to a tissue sample containing large numbers of tubules

because of a certain amount of inhomogeneity in the tubules of most specimens. After observing a sufficiently large number of such preparations, the following conclusions were reached: (a) In any preparation there are always some tubules which never take up any dye. Presumably these have been traumatized in the process of removal from the fish and hence may be disregarded in evaluating the specimen. (b) If a mass of tubules has not been well separated by gentle teasing, the tubules in the interior usually take up dye more slowly than those in direct contact with the external medium. Therefore, in time-controlled experiments, care must be taken to achieve good separation of the nephrons. This is particularly important when the effects of inhibitors are being studied, since a tubule isolated in the interior of a tightly packed mass is also protected from exposure to the full concentration of the depressing agent which is acting on the more peripheral tubules. (c) Any preparation which is concentrating phenol red eventually loses its powers of active transport and discharges its accumulated dye. This degeneration does not usually occur until after one or two hours at 28°C. As will be shown, these tubules are performing work against large concentration gradients. In all probability, the supply of some essential intermediate becomes exhausted in the course of this expenditure of energy which takes place in a medium containing only inorganic salts. Because of this effect, only readings taken during the buildup of the dye concentration to its maximum value in the tubule and its maintenance at this point were regarded as the significant part of any experiment. There was usually no difficulty in maintaining peak activity in a preparation for 20 or 30 minutes. During the first 40–50 minutes of an experiment, the majority of the tubules of any preparation showed the same degree of activity, and this value was taken to represent the state of that particular specimen. The readings taken by the various experimenters on the same specimen agreed closely, and curves obtained on tissues taken from different fish on different days usually showed quantitative agreement within the limits of precision of the method. Occasionally a

fish was found whose tubules showed an abnormally low absolute activity, but even in such specimens the relative effects of the different agents employed in the course of the study were the same as in the rest of the group.

EXPERIMENTAL RESULTS

1. Typical dye-accumulation curve in balanced salt solution

In agreement with the results of Forster ('48) and Forster and Taggart ('50) rapid uptake of phenol red was found to

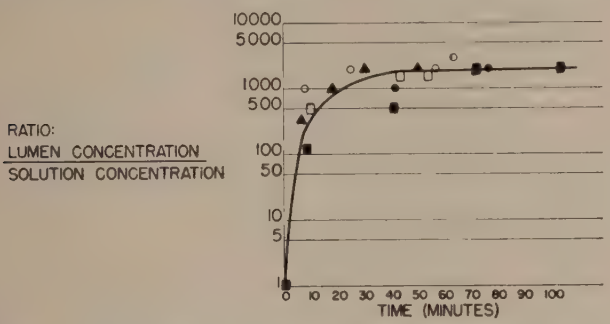

Fig. 1 Ratio of phenol red concentration inside the tubule lumen to that in the external solution, for flounder kidney suspended in the balanced ionic medium of Forster and Taggart ('50) at 28°C. The concentration of phenol red in the solution was 0.01 mg/cm³. Each set of points represents an experiment on a different fish on a different day.

occur in the tubule lumen when kidney tissue is placed in a balanced salt solution containing the following components: NaCl, 0.13 M; KCl, 0.0025 M; $CaCl_2$, 0.0015 M; $MgCl_2$, 0.001 M; $NaHCO_3$,[6] 0.02 M; NaH_2PO_4, 0.0005 M; phenol red, 2.8×10^{-5} M (0.01 mg/cm³). For convenience we shall refer to this solution as the "balanced salt medium." Figure 1 presents a typical curve showing the time course of dye accumulation in the lumen of a tubule maintained in the balanced medium at 28°C. It will be seen that these tubules are capable of excreting

[6] The concentration of $NaHCO_3$ employed exceeded that recommended by Forster because it was found that the more alkaline solution decreased the tendency for the dye to change over to the acid color inside the tubule.

phenol red in the lumen against a concentration gradient greater than 2000:1. The curves obtained from different specimens on different days were remarkably parallel. The lowest concentrating power ever observed for the conditions described in figure 1 was 1000 times, while values as high as 4000 were not uncommon.

2. *Effect of varying the concentration of phenol red*

Uptake curves carried out in the balanced salt medium with a series of different dye concentrations demonstrated that

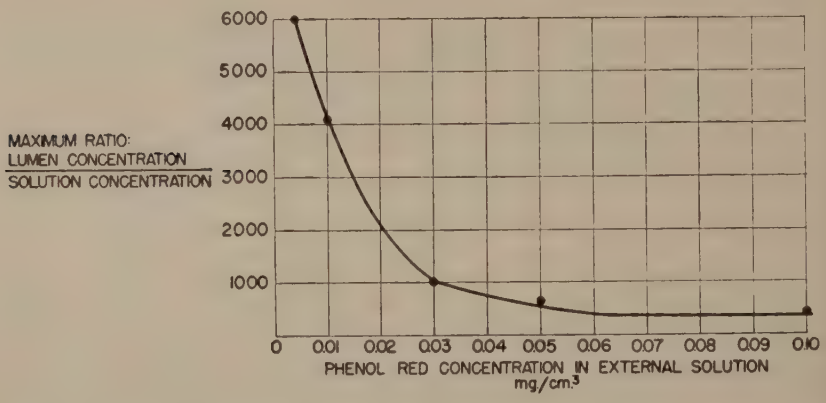

Fig. 2 Ratio of maximum phenol red concentration in tubule lumen to that in the outer solution for varying concentrations of the dye in the medium.

variation in the concentration of phenol red in the external solution from 0.003 to 0.10 mg/cm³ had almost no effect on the maximum dye concentration finally achieved in the lumen. Thus, the relative concentrating power of the tubule decreases as the absolute concentration of phenol red in the medium increases (fig. 2). It may be concluded then that the concentrating ability of the tubule depends upon the actual number of molecules available for transport, rather than on the thermodynamic free energy change alone, which is determined only by the ratio of dye concentrations inside and outside the tubule.

3. Demonstration of a mechanism for accumulation of dye in the cells

Microscopic examination of the transport of phenol red in tubules suspended in the balanced salt medium of Forster and Taggart ('50) reveals that even when a high concentration of dye has accumulated in the lumen, the cells usually exhibit no uptake of dye whatever. In fact, they are even less colored than the surrounding solution. Since actively transported dye can only reach the lumen by passage through the cells, the mechanism for moving dye molecules from the cell into the lumen, under the given conditions, must operate at a faster rate than that which brings the dye into the cell. Both processes go on in a medium containing only inorganic salts. Hence, it was of interest to investigate the roles of the individual ions on these two stages of the process.

Experiments in which the concentrations of the various ions were systematically varied revealed the following facts: If K^+ alone is omitted from the medium, no accumulation of dye occurs in either the cells or the lumen. If the K^+ concentration is increased 4 to 6 times over that in the balanced medium, the maximal concentration achieved in the lumen is not affected appreciably, but a small accumulation of dye in the tubule cells often occurs as well. If Ca^{++} alone is omitted from the balanced medium, the ability of the tubule to concentrate phenol red in the lumen is lost. In such preparations also, however, a small accumulation of dye usually occurs in the cells.[7]

These observations lend themselves to explanation by the following provisional hypothesis: Potassium is required by the mechanism which first transports the dye into the tubule cells (hereafter to be called Step I). Therefore, in the absence of K^+ the preparation is completely inert. Ca^{++} is not necessary for this first step, but is required for the process in which the dye is transferred from the interior of the cell into the lumen (Step II). Hence, in the absence of Ca^{++}, no dye can be transported into the lumen, but, since Step I can still function,

[7] Because of the high level of HCO^-_3 employed, it was not possible to study the effects of increased concentrations of Ca^{++}.

dye will accumulate in the cells to a concentration determined by the chemical potential which Step I, acting alone, can bring to bear. The process may be represented as follows:

Step I: Phenol red + K^+ + cell $\rightleftharpoons$ Phenol red in cell
Step II: Phenol red in cell + Ca^{++} $\rightarrow$ Phenol red in lumen

According to this scheme one would expect that simultaneous increase of the concentration of K^+ and decrease in the concentration of Ca^{++} would cause a large accumulation of phenol red in the cell, since the first step would be accelerated, while the second would be decelerated. This behavior was observed experimentally. Table 1 shows the result of a typical experiment:

TABLE 1

Maximum concentration of phenol red relative to that in the outer solution, achieved in the tubule lumen and cells, respectively

	IN BALANCED SALT MEDIUM $[K^+] = 0.0025$ M: $[Ca^{++}] = 0.0015$ M	IN HIGH K-LOW CA MEDIUM $[K^+] = 0.015$ M: $[Ca^{++}] = 0$
In lumen	1000	None detectable
In cells	0	500

The phenomenon was completely reproducible in a series of more than 30 experiments. In some trials, a 2000-fold concentration of dye in the cells could be achieved. In the case of high K-low Ca medium, it is not possible to conclude that dye is completely absent from the lumen of the tubule, since the large amount of dye which collects in the cells tends to obscure the condition of the inside of the cylinder. It is certain only that in the presence of high K and low Ca, a high degree of dye concentration in the cells is achieved, and dye transport to the lumen, if it occurs at all, is much less than that which obtains in the balanced ionic solution. This difference in the effects produced by the two media is particularly striking in those tubules which can be viewed end-on when the lumen and the cell layer appear as two concentric circles. In the balanced solution one sees a red center and white periphery, while in the case of the high K-low Ca, the periphery is red and the central portion much paler.

4. Kinetics of the K-Ca effect

The foregoing experiments suggest that a simultaneous increase of K and decrease of Ca in the medium permits isolation of what may be the first step of tubular excretion; i.e., the initial entry of phenol red into the cell. In order to test this tentative conclusion, the effects of various factors on this reaction were measured quantitatively.

(a) Effect of varying the concentration of K^+. Starting with the normal balanced medium, if the concentration of K^+ is gradually increased, dye deposition occurs only in the lumen as long as the K^+ concentration remains less than 0.0085 M.

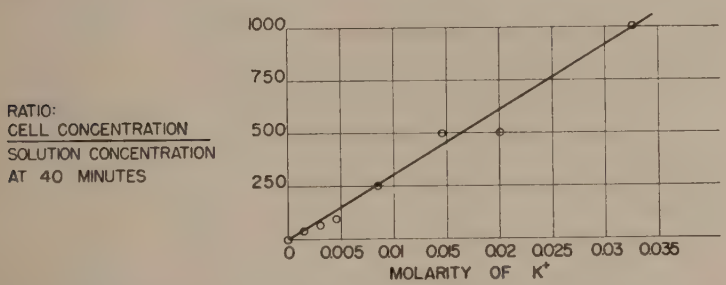

Fig. 3 Effect of varying the $[K^+]$ in a medium lacking Ca^{++}, on the rate of concentration of phenol red in the tubule cells. The amount of dye accumulated at the end of 40 minutes has been used as the basis for comparison.

Beyond this value dye appears in the cell as well. This mixed deposit of phenol red in cell and lumen can regularly be observed with K^+ concentrations as high as 0.02 M, nearly 10 times the normal concentration.

In the absence of Ca^{++}, the effect of varying the concentration of K^+ is simpler. Since no dye ever appears in the lumen, only the single process of dye deposition in cells can occur. Under these conditions, the rate of accumulation of phenol red in the cells is a linear function of the concentration of K^+ in the medium as shown in the curve of figure 3. This direct dependence of the rate of the first reaction on the concentration of K^+ would be expected on the basis of the scheme proposed.

(b) Effect of competitors for the phenol red transport system. Forster and Taggart ('50) demonstrated that tubular transport of phenol red to the lumen of the flounder kidney is inhibited by specific substances like PAH (p-aminohippurate) and penicillin, which compete for the same transport system in mammalian kidney. The effects of PAH on the cellular accumulation of phenol red in the high K-low Ca medium were tested to determine whether this process would also display competitive inhibition. Failure to demonstrate such a response would mean that cellular accumulation of the dye is not related to the active transport mechanism, but is due rather to a non-specific reaction with other cellular components.

Both PAH and diodrast — also a specific competitor for tubular transport —inhibited the cellular accumulation of phenol red in the high K-low Ca medium. The concentrations of these substances required to produce this inhibition were the same as those necessary to inhibit excretion into the lumen in the balanced ionic medium. Table 2 summarizes a typical experimental series. This parallelism in action of these competitive agents supports the presumption that the cell-concentrating mechanism here described is part of the normal excretory process.

(c) Other effects. The ability of a tubule to concentrate phenol red in the cells or lumen under the influence of the appropriate ionic mixture was found to be reversible, provided that exhaustion effects had not set in. Thus, a tubule in which dye had accumulated in the cells could be converted to one displaying concentration only in the lumen, by transfer from the high K-low Ca medium to the balanced one.

The transport of dye into the cells in the high K-low Ca medium also displayed low-temperature inhibition, and exhaustion effects like those characteristic of transport into the lumen. Exhaustion of the capacity to concentrate dye in the cells occurs after approximately an hour in the high K-low Ca medium at 28° and the accumulated dye becomes discharged. Moreover, tissue which has exhausted its cellular concentrating power in a high K-low Ca solution, and is then transferred to

TABLE 2

Demonstration that PAH inhibits the accumulation of phenol red in the cells as well as in the lumens of kidney tubules

	Ca^{++} PRESENT NORMAL K^+						Ca^{++} ABSENT THREE TIMES NORMAL K^+					
Concentration of PAH: (mg/cm³)	0		0.05		0.10		0		0.05		0.10	
	Cell	Lumen	Cell	Lumen	Cell	Lumen	Cell	Lumen	Cell	Lumen	Cell	Lumen
Concentrating power for phenol red after 38 min.	0	750	0	250	0	62	500	0	62	0	0	0

the balanced electrolyte medium, is found also to be unable to carry out normal transport into the lumen. Thus, whatever the factor which is responsible for the exhaustion phenomenon, it affects both types of phenol red accumulation.

DISCUSSION

A two-stage scheme for tubular active transport was first proposed by Shannon ('39). Franck and Mayer ('47) recently presented a detailed physico-chemical picture of how active transport could be accomplished, which also involves two distinct stages of this type. However, unequivocal isolation of the step in which the transported material accumulates in the cell has not previously been accomplished. Forster ('48) and Forster and Taggart ('50) found no cellular accumulation of phenol red in the tubules of the frog, sculpin, and flounder under conditions of normal transport, nor in the flounder, under the inhibiting influence of low temperature, Hg^{++}, iodoacetate, 2, 4-dinitrophenol, PAH, penicillin, and carinamide. Intracellular accumulation of phenol red and related compounds in embryonic chick and mammalian kidney tubules under conditions of high dye concentrations (for example, 0.25 mg/cm^3), has been reported by Chambers and Kempton ('33), and Cameron and Chambers ('38). However, in these experiments the tubular urine also contained large amounts of dye, so that the second step of excretion was not blocked, at least to the extent which is achieved in the high K-low Ca medium.[8] Recently Josephson and Kallas have observed cellular accumulation of diodrast in the tubule cells of the rabbit kidney also under conditions of tremendous overload of this substance in the plasma, which they also explained on the basis of a two-step mechanism. The present demonstration of a concentrating mechanism in the cells, the operation of which does not require an overload of the transported substance in the medium, con-

[8] In the studies of Chambers and his co-workers the cells were usually stained yellow instead of red because of the higher intracellular pH, a condition which was prevented in the present work by the excess of $NaHCO_3$ in the medium.

stitutes strong support for the two-stage mechanism of tubular transport.

The evidence that the process resulting in accumulation of phenol red in tubule cells in the high K-low Ca medium is indeed a part of the normal transport system, rather than a nonspecific staining action, may be summarized as follows: The effect is inhibited by the same specific molecular competitors in the same concentration range required to block normal transport. Tubules which have exhausted their ability to carry on this cellular concentration are also no longer able to carry on transport into the lumen. The cellular dye accumulation exhibits the same low-temperature inhibition as does the normal process. Finally, this cellular-concentrating mechanism has at least some specificity for the tubule cells, since such concentrating action was never observed in the cells of adjacent tissues which often were present in the same experimental vessel.

The data presented here are not sufficiently extensive either to support or invalidate the assumptions of Shannon or Franck and Mayer about the specific characteristics of the separate reactions involved in active transport. It is of interest to note that a two-step scheme of the type involving intermediate binding of the dye to some cellular component would display at least qualitatively the kind of dependence on phenol red concentration which was observed here (fig. 2), since, as the concentration of the transported substance increases, the limited number of phenol red-binding sites within the cell would tend to become saturated. On the other hand, if active transport were carried on by means of a cycle of simple permeability changes alone, the relative concentrating power of the tubule would be independent of the dye concentration in the external medium.

The use of the high K-low Ca medium would appear to provide a tool for isolating the separate stages of the process and examining their kinetics in detail. Although the quantitative technique described in this communication is so crude as to permit errors as great as 50 or 100% in any reading, the mag-

nitude of the concentrating action is so great (6000:1) that a 2:1 error in any single measurement does not greatly diminish the significance of the results. With better temperature control, even better accuracy can undoubtedly be achieved.

The present experiments permit estimation of the minimum thermodynamic work against concentration gradients which the tubule can perform. When the outer solution contains a phenol red concentration of .01 mg/cm^3 or 2.8×10^{-5} M, a 4000-fold concentration of dye can be achieved in the lumen in approximately 30 minutes at 28°C. Assuming constant activity coefficients, the free energy change is:

$$\Delta F = + nRT \ln \frac{a}{a'}$$

$$= + n \times 2 \times 301 \times 2.303 \times \text{Log} \frac{4000}{1}$$

$$= + n\ (5000)\ \text{calories}$$

In 1 cm of tubule, the volume of the lumen is $\pi(0.95 \times 10^{-3})^2 \times 1 = 2.8 \times 10^{-6}$ cm^3, or 2.8×10^{-9} l. The dye concentration inside the lumen is $4000 \times 2.8 \times 10^{-5}$ or 0.11 M. Therefore, in 1 cm length of tubule (which contains approximately 104 cells) the number of osmoles, n, is $2 \times 2.8 \times 10^{-9} \times 0.11 = 6.2 \times 10^{-10}$. Therefore, ΔF per centimeter length of tubule is $5000 \times 6.2 \times 10^{-10} = 3.0 \times 10^{-6}$ calories. Since the process is undoubtedly far from 100% efficient, the actual energy expenditure must be considerably greater than this figure.

SUMMARY

1. A method is described for measuring quantitatively the uptake of phenol red in the isolated kidney tubules of the flounder. Data are presented showing the time course of phenol red accumulation in the lumen of such tubules when a balanced salt medium is employed. Under optimal conditions the dye concentration achieved in the lumen is 4000–6000 times as great as that in the external solution.

2. The relative concentrating power of the tubule for phenol red decreases as the concentration of the dye in the external solution increases. Hence, some process within the tubule appears to reach a saturation point at high phenol red concentrations.

3. If the salt solution bathing the tissue is relatively low in K^+ and high in Ca^{++}, accumulation of phenol red is limited to

the lumen, and none is detectable in the cells. In salt solutions containing a high K^+ concentration and no Ca^{++}, the dye accumulates in high concentration in the tubule cells, with little or none appearing in the lumen.

4. The accumulation of dye in the tubule cells in high K and low Ca media is inhibited by specific agents like PAH and diodrast which compete for the normal phenol red tubular transport mechanism. PAH competition exhibits the same concentration relationships for cellular and lumen accumulation; therefore, it is proposed that the mechanism which leads to accumulation in the cells is part of the normal transport mechanism.

5. In the complete absence of K^+ no phenol red uptake in cells or lumen occurs. In the absence of Ca^{++}, the dye is taken up only in the cells, but not in the lumen. Moreover, under the latter condition, the rate of accumulation of dye in the cells is directly proportional to the concentration of K^+ in the medium.

6. The above phenomena are explicable on the assumption that tubular transport involves two steps: transposition into the cell which requires K^+, and transposition from cell to lumen, which requires Ca^{++}.

The extremely helpful criticisms and suggestions of Drs. Homer W. Smith and Ray P. Forster are gratefully acknowledged.

LITERATURE CITED

CAMERON, G., AND R. CHAMBERS 1938 Direct evidence of function in kidney of an early human fetus. Am. J. Physiol., *123:* 482.

CHAMBERS, R., AND R. T. KEMPTON 1933 Indications of function of the chick mesonephros in tissue culture with phenol red. J. Cell. and Comp. Physiol., *3:* 131.

CROSS, R. J., AND JOHN V. TAGGART 1950 Renal tubular transport. The accumulation of p-aminohippurate by rabbit kidney slices. Am. J. Physiol., *161:* 181.

FORSTER, ROY P. 1948 Use of thin kidney slices and isolated renal tubules for direct study of cellular transport kinetics. Science, *108:* 65.

FORSTER, ROY P., AND JOHN V. TAGGART 1950 Use of isolated renal tubules for the examination of metabolic processes associated with active cellular transport. J. Cell. and Comp. Physiol., *36:* 251.

FRANCK, JAMES, AND J. E. MAYER 1947 An osmotic diffusion pump. Archives of Biochem., *14:* 297.

SHANNON, JAMES A. 1939 Renal tubular excretion. Physiol. Rev., *19:* 63.

THE IMMEDIATE EFFECTS OF POTASSIUM ON RESPONSES OF SKELETAL MUSCLE [1, 2]

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FOUR FIGURES

One of the more obscure fields of physiological research is concerned with the effects of potassium on muscular activity. Although many definite alterations of both excitation, contraction, and even metabolism are known to occur when muscle is subjected to abnormal concentrations of this ion (e.g., Hegnauer, Fenn and Cobb, '34; Solandt, '36; Chao, '37; Carleton, Blair and Latchford, '38; Smith and Solandt, '38; Fenn, '40; Höber, '45), their interpretation is difficult. This is due partly to the duality of the K effects, for evidently depending on concentration and duration of action this substance may either depress or enhance the various functions it modifies. Furthermore, particular uncertainty is encountered in those cases in which mechanical responses are altered, since it is not generally clear whether the K has penetrated into the fibers and thus possibly acted directly on the contractile system or whether the mechanical disturbances result indirectly from effects engendered in the superficially located excitatory system.

[1] These studies were aided by a contract between the Office of Naval Research, Department of the Navy and the New York University (NR 113–300).

[2] Preliminary notices of some of this work have appeared as follows: Fed. Proc., 8 (no. 1): 177, 1949, and 9 (no. 1): 68–69, 1950.

[3] This paper is based on the Master's thesis of Dr. Kahn, whose present address is: Department of Physiology and Biophysics, Georgetown University School of Medicine, Washington, D. C.

In the current research we deal with the effects of various concentrations of K on the mechanical latent period, the peak tension output, and the action potential of isometric responses of frog skeletal muscle during the first hour or two of the action of the K. We have paid special attention to the modifications which appear within the first few minutes after the K begins to act, for under these conditions, and even though the K has been augmented considerably above normal concentration, the time is too short for penetration of the K into the muscle fibers. Hence it becomes possible to distinguish effects which are directly on the surface of the fibers, i.e., on the excitatory system, from those which involve K-penetration, and therefore signify action of K directly on the inner contractile system.

METHOD

All experiments were performed on sartorius muscles of medium sized Rana pipiens, which after excision were first routinely equilibrated for an hour in two changes of oxygenated Ringer's solution buffered with phosphates at pH 7.2. The piezoelectric, cathode-ray oscillographic method served to record the latency mechanical changes of isometric twitches and simultaneous optical myography registered the initial and peak developed tension of each response (Sandow, '44). Stimulation was effected by the transverse, massive electrode method (Brown and Sichel, '36) as developed for use on whole muscle (Sandow, '47a). This procedure for stimulation has certain general advantages over conventional wire-electrode methods which make it especially desirable for the type of experiments of our present research. It causes essentially simultaneous initiation of the mechanical response of all segments of the muscle length (Sandow, '48); this precludes complications due to mechanical wave effects which develop from the localized application of a shock through wire electrodes, and this results in a much larger depth of latency relaxation. Furthermore, the fact that the massive method requires stimulating the muscle while in an electrolyte bath

provides for continuous maintenance of the tissue in a test medium during the entire course of an experimental run.

In view of the very low electric impedance of the massive electrode bath (of the order of 10 ohms), stimulating shocks must be of high current density in order to excite the muscle. Such shocks were obtained by using a thyratron (884) discharge circuit with 90 volts on the plate of the tube. The thyratron at rest was biased to cut-off and its discharge was controlled by the keys, K_1 and K_2, of a Lucas rheotome. When K_1 was opened, the bias was removed and the thyratron abruptly began to pass current and this was terminated at a controllable later instant by the opening of K_2 which separated the thyratron from its plate supply. The pulses produced by this stimulator were essentially square-wave with a maximal current strength of about one ampere, and, although the shock duration could be varied, it was held constant at 0.17 ms in all our experiments. The shock was led off from a 20 ohm potentiometer placed in the cathode circuit of the thyratron, and it was arranged to be of slightly supermaximal physiological strength for direct stimulation of the muscle equilibrated to normal Ringer's solution.

Since, in general, the muscles were not curarized, the choice of a direct stimulus of proper strength could not be simply determined by observation of tension output. But such a stimulus could always be chosen by means of certain criteria characteristic of the latency mechanical behavior described in detail in an earlier paper (Sandow, '44). Although this procedure did ensure that the muscles were responding to direct stimulation, account was taken of the fact that they were not curarized and that therefore some features of their behavior under the influence of potassium might be due to events localized in the intramuscular nerve fibers and at the neuromuscular junctions. Hence, control experiments were performed involving both mechanical and action potential behavior of maximally responding curarized muscles. The results of such tests were identical with those obtained from the directly stimulated uncurarized tissues, and we therefore

conclude that our general procedure yielded results that are attributable to the action of potassium only on the muscle fiber.

In any run involving recording of the mechanical changes, the muscle was mounted in normal Ringer's solution in the massive electrode chamber and put under a standard initial tension of 3.0 gm. Then, after determination of the standard supermaximal shock strength, photographic records were made of the responses of 4 normal maximal twitches evoked at one-minute intervals. The averages of the relevant parameters of these responses served as the normal controls. The normal Ringer's was now replaced by an experimental solution containing a definite excess of KCl, and a series of responses were recorded, the stimuli for these (with an exception to be noted later) being of the same strength and duration as those used for the normal tissue. Generally, responses were obtained at one-minute intervals during the first 8 to 10 minutes of the run, and thereafter readings were taken at greater time separations and for a total duration so chosen as to demonstrate the essential course of the characteristic changes. The experimental solutions used in separate tests contained the following concentrations of potassium expressed as mg %: 18.0, 23.0, 28.1, 38.3, 48.4, and 68.7; and from three to 6 muscles were tested at each concentration. Controls for these runs consisted of muscles treated similarly as far as the sequence of responses was concerned, but in contact throughout with only standard Ringer's solution (7.8 mg %). During the course of any of the above experiments care was taken to maintain the resting tension at the standard 3 gm value; however, only occasional minor adjustments had to be made, chiefly in connection with the contracture that developed for a short time in each of the muscles placed in a medium containing 38.2 mg % or more of the potassium. All experiments were performed at a temperature of 24.5°C. controlled by a water bath constant to $\pm$ 0.01°C.

In all cases, each of the experimental media were formed by simply adding the required amount of 5%KCl solution to

100 cm^3 of normal Ringer's solution. We did not remove from each medium an amount of NaCl osmotically equivalent to that of the added KCl, since the latter, at most, was too small to effect any significant osmotic imbalance. Nevertheless, we performed several control experiments in which muscles were tested while immersed in a Ringer's solution that was normally balanced in respect to its cations, but made hypertonic to a degree equivalent to the presence of 48.4 mg % K. Under this condition certain slight effects were noted, especially on the depth of the LR, which were evidently consequences of the osmotic imbalance; but they were quite different from those we have observed under the influence of the KCl-enriched solution. We therefore conclude that our essential results are not attributable to osmotic disturbances resulting from the presence of the excess KCl.

In the procedure for obtaining action potentials, each muscle was soaked in its experimental solution in a beaker mounted in the usual constant temperature bath. To record the electrical response, the muscle was temporarily removed from its solution and set up in contact with the wire electrodes in a moist chamber as employed in conventional action potential recording. Stimuli were thyratron controlled condenser discharges having a time constant of 0.2 ms. They were always of the same slightly supermaximal strength as determined for the normal Ringer's equilibrated tissue, and were applied to the nerve-free pelvic region of the muscle so as to ensure excitation of only the muscle fibers. The magnitude of the action potential was measured by the peak deflection of the first limb of the diphasic response. The concentrations of the potassium in the test media and the essential sequence of responses obtained for the muscles soaked in each were the same as those used for investigating the mechanical changes.

Muscles immersed in solutions containing 38.3 mg % or greater concentrations of potassium ion immediately developed visible spontaneous twitches which appeared for about 30 to 60 seconds; and as previously mentioned some contracture also arose and then disappeared. Such spontaneous

activity in no way interfered with the carrying out of our experimental procedures.

RESULTS

A diagram of a typical record of the mechanical responses is shown in the inset of figure 1. The oscillographic sweep starts at the extreme left at the instant that the shock begins; the initial downward rectangular deflection corresponds to the shock used and measures its strength and duration. During the shock interval there is no mechanical response of the muscle and this state is maintained after the termination of the shock until the sweep is about half over when there occurs the sigmoid shaped large downward deflection representing the latency relaxation (LR). The LR is terminated quite sharply as the trace begins to move rapidly upward in the very earliest phase of positive tension development. To the right of this record is seen a pair of lines corresponding to the optical myographic record. The upper line indicates the resting muscle tension and the lower one measures the twitch peak tension output. L_R is the time to the beginning of the LR; L, the duration of the mechanical latent period; R, the depth of the LR; and T, the value of the peak developed tension.

Latency changes. The average changes of R with time of immersion in each of the test potassium media are demonstrated in figure 1. All values of R are compared on a percentage basis, with the behavior before exposure to each potassium-enriched medium taken as 100%. For the muscles maintained in normal Ringer's (7.8 mg % K), R remains essentially constant. But for all media with greater potassium concentrations, two general effects are noted. First, there is a rapid increase in R during the first one to 5 minutes, and second, after a variable period of more or less maintenance of this increase, R falls. In general, there are several relations between the potassium concentration and the corresponding course of the rise and decline in R: (1) the greater the concentration, the more rapid the initial increase; (2) the largest

increase in R seems to be fairly constant (90 to 100%) for concentrations up to 28.1 mg %, but for greater excess K this increase is larger, the more the K (excepting, however, the 68.7 mg % behavior — an anomaly to be discussed later); (3) the interval during which the peak increase is maintained is less, the greater the K-concentration; (4) the rate of fall of

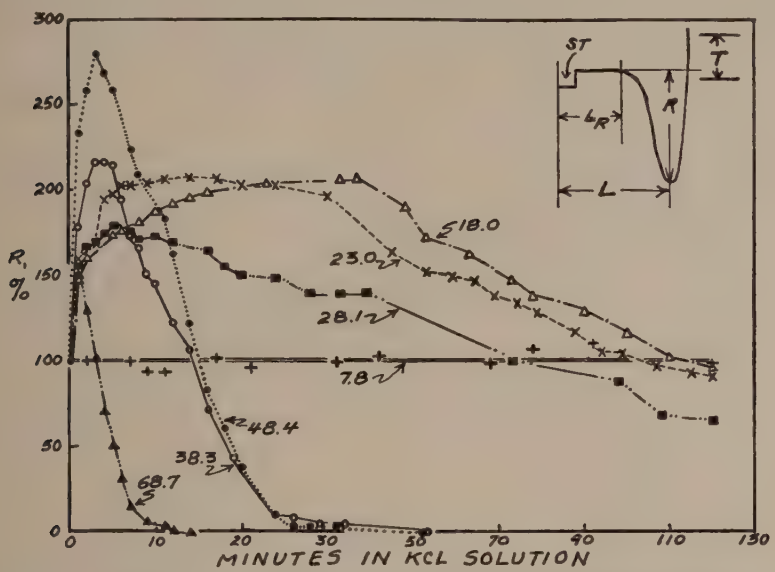

Fig. 1 Change in R, the depth of the latency relaxation, as a function of time of soaking of muscles in K enhanced solutions. Note the change in time-scaling after the 30-minute point. The number labelling each curve gives the K concentration in mg %. 7.8 mg % is normal. *Inset* gives a diagram of the photographed records showing the latent period changes and the initial and peak tensions. See text for definition of symbols.

R is rather uniformly low for concentrations up to 28.1 mg % and, furthermore, for this concentration range even after two hours of action of the excess K, the smallest value of R is relatively high; but for the greater concentrations R falls at a very high rate and in each case it is relatively soon reduced to zero.

Measurements were also made of the temporal variables, L_R and L under the action of the various concentrations of

potassium, but the results were erratic. However, at least in certain experiments a trend was observed, more clearly shown by L than by L_R, which indicates that these parameters decreased during the initial phase of exposure to a given potassium solution, when R and T were increasing, and thereafter they increased as R and T diminished, becoming even greater than normal when R and T fell below their values in normal Ringer's.

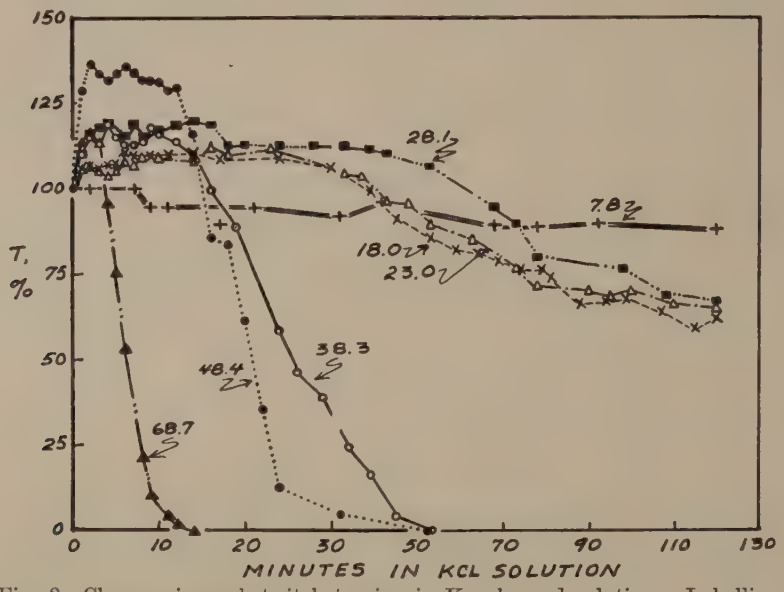

Fig. 2 Changes in peak twitch tension in K enhanced solutions. Labelling of curves as in figure 1.

Tension changes. The average variations with time of the peak isometric tension (T) developed by the same muscles (as those just studied for the R-changes) for the same set of potassium concentrations are demonstrated in figure 2. The method for plotting these alterations is the same as that used for the R-changes. It will be noted that even for the normally treated muscle, T falls gradually and slightly with time; this seems to be characteristic behavior of muscle in a normal Ringer's bath. But under all conditions involving augmented

potassium concentration these curves show that the muscles developed potentiated tension output during the initial period of action of the potassium and that this was followed by a period of declining contractile strength. This parallels the changes in R; but the greatest increases in T are several times smaller than those of R, and the time course of the T and R changes do not exactly match (compare, e.g., the results with 48.4 mg % K). But a point to be stressed is that the

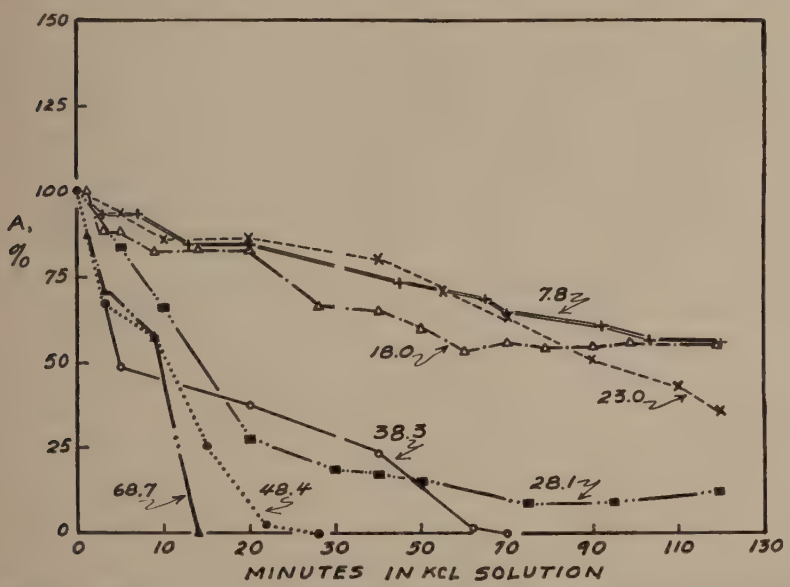

Fig. 3 Changes in action potential height in K enhanced solutions. Curves labelled as in figures 1 and 2.

decreases in T, like those of R, show rates of fall and final diminutions which are moderate for K-concentrations of 28.1 mg % and less, but which are much more pronounced for the concentrations of 38.3 mg % and above.

Action potential changes. The variations in magnitude of the action potential are presented in figure 3. In all cases, the action potential decreased progressively from the moment a muscle was placed in a given medium. However, the rate of decrease of A sharply increases when the medium contains

K in 28.1 mg % concentration or greater. This behavior is comparable to that already discussed for R and T, although these variables first exhibit their greatly increased rates of fall at a definitely higher level of K, i.e., at 38.1 mg %. It is important to note that, although the muscles used for the action potential studies were stimulated by wire electrodes in moist air, they nevertheless showed the same T and R modifications as those massively excited.

K, the active ion. Throughout the preceding, we have assumed that our results are due to augmentation of the K-ion concentration and not to the chloride — an interpretation made by other workers in comparable experiments. To ensure the validity of this interpretation, we have performed several experiments in which the potassium concentration was raised in the Ringer's solution by addition of potassium sulphate or potassium nitrate. The amount of the sulphate that could be added was limited by the formation of a precipitate with the calcium of Ringer's; but enough of this salt could be introduced to give a potassium concentration as high as 48.4 mg % without obvious formation of the precipitate. In such media, despite the change of anion, the behavior of the muscle tested in the standard procedure was identical to that produced by equivalent amounts of potassium chloride. In the experiments utilizing potassium nitrate, there was no limitation of the amount of salt that could be placed in the Ringer's solution. With the lower concentrations the results again were typical of the potassium chloride effects. But with higher concentrations of the added nitrate, usual results were obtained as with potassium chloride; but superimposed upon these were extraordinarily high augmentations in tension output, evidently due to the presence of the nitrate ion. A preliminary report of the results of certain experiments dealing with this nitrate effect is given elsewhere (Kahn and Sandow, '50). Of immediate interest, however, is the fact that the effects we have obtained in experimental media enriched with potassium chloride can be in essence duplicated with the use of potassium salts of sulphate and

nitrate as well. These results thus prove that the common effects of these salts are due to the presence of the common K-ion, and not to the associated anion.

Excitability changes and their relation to mechanical responses. Earlier work, e.g., by Chao ('37) and by Carleton et al. ('38), has shown that in general a given concentration of K causes an immediate decrease in threshold; this is later reversed and the threshold then becomes greater than normal. Using massive stimulation, we have repeated such experiments and have obtained similar results. The time-course of these excitability changes is in general like that of the R and T changes for the same concentration of K. Our R, T, and A variations, however, have been always obtained in any one experiment in response to shocks which were slightly supermaximal for the particular muscle in normal Ringer's solution. Since the excitability of K-treated muscles varies, the question arises as to whether we were obtaining maximal mechanical responses from each of our treated muscles throughout its experimental run; i.e., is it possible to account for the different alterations in the responses by changes in recruitment of fibers?

During the initial period (i.e., the time interval during which the R and T values are above normal) the increase in excitability of the fibers could not have led to the augmented R and T outputs by recruitment of fibers; for the supermaximal shocks we used ensured that the responses were truly maximal even for the tests made on each muscle in normal Ringer's solution. We therefore conclude that these enhanced outputs are due to an increased contractile output of each of the muscle's fibers, induced somehow by the immediate action of the K. It might seem that the progressive decreases in the action potential observed during this period indicate that progressively more of the fibers were failing to respond. This could hardly be true in view of the concurrent increase of excitability. Furthermore, a decrease in the action potential of a whole muscle treated with excess K might be attributed to smaller than normal action potentials in all of the available

fibers, as is indicated by the decrease in action potential height in similarly treated single nerve axons (e.g., Hodgkin and Katz, '49).

The behavior of the muscles after longer action of K (especially in concentrations of 38.3 mg % and greater, in which R, T, and A show rapid and pronounced decreases in output) involve responses of muscles with thresholds far above normal. The shocks which were evoking maximal responses during the earlier period of K-action might during this later period be causing submaximal responses. To test this possibility, several experiments, particularly with the 48.4 mg % concentration of K, were done in which the maximal shock strength was determined as a function of the time of action of K. It was found that maximal tension output required shocks that were as much as double those of the standard strength. However, even so the added tension output obtained at any moment was only a few per cent above that evoked by the standard shock at that time. From these results we conclude that the responses obtained with our standard slightly supermaximal shocks were not quite maximal. Nevertheless, it must be noted that the increment in T obtained by using shocks of twice the standard strength was not very great, i.e., the output was still far below that produced in maximal responses of the muscle in normal Ringer's solution. This diminution in output, unfortunately, cannot be accounted for unequivocally. It may be due to some, and, as time passes, progressively more, of the fibers having been made completely inexcitable by the relatively long K-action, or to smaller mechanical outputs from fibers still excitable; or it may be due to a combination of these actions.

Still another point of interest in connection with the above discussion is our observation that even though a muscle has become completely inexcitable (i.e., can no longer generate action potentials) due to K-action, it will still respond mechanically in a sort of twitch-like contracture if it is subjected to a very intense massive shock. In the present experiments, the shock strengths at their greatest were still not intense

enough to evoke this type of response. Detailed studies of this kind of mechanical change have been made by us and they will be reported elsewhere.

Reversibility tests. We have done a small number of experiments to determine whether the mechanical changes which appear after action of K for a few minutes were reversible. In several tests, the muscles were exposed to a Ringer's solution containing 23 mg % K for 10 minutes, at the end of which time the usual increases in R and T were obtained, and then normal Ringer's was restored to the chamber. Although the tension output now reversed to the values characteristic of a muscle immersed throughout in the normal medium, R did not reverse completely; even after two and one-half hours the value of R, though it had declined, was still about 75% above normal. Had the solution with the enhanced K continued to act on the muscle, R would have fallen to smaller than normal values. In another pair of tests the muscles were placed in a solution containing 48.4 mg % K for three minutes (the time for maximum enhancement by this solution) and then tested in Ringer's. And, here also, T showed a reversal to expected values for continued immersion of the muscle in Ringer's solution, but R (initially increased by about 200%) after two and one-half hours was still about 125% above normal.

These results indicate that the increase in R resulting from a brief exposure of a muscle to a K-concentration which is greater than normal, tends to decrease very slowly when the muscle is replaced in ordinary Ringer's solution; i.e., under these conditions the enhancement of R by K is relatively irreversible. The tension output tested under similar conditions seems to be quite reversible. To test further the reversibility behavior of R, one experiment has been done in which a muscle was placed for two minutes in a 48.4 mg % K medium, after which it was quickly washed in normal Ringer's for final study. In this case, not only was the initial tension augmentation completely reversed but that of R was also. Thus it seems that the enhancing effect of K on the latency relaxation can be completely reversed, but that this requires more thorough

washing than does the reversal of the tension increase. Although it is evident that more work needs to be done on these reversibility effects, the results of the few experiments reported here are clear and of sufficient interest to place on record at this time.

DISCUSSION

Certain features of our results concerning the tension output and the action potential have been in essence reported in previous work (e.g., Fenn and Cobb, '34; Hegnauer, Fenn and Cobb, '34; Baetjer, '35; Brown, '37; Brown and von Euler, '38; Guttman and Cattell, '40; Walker, '47a and b; and in reviews by Fenn, '40 and Höber, '45). The general conclusion that emerges from all this earlier work is that muscular mechanical responses may be either enhanced or depressed by K depending on the concentration and length of time of action of the ion. But in the interpretation of these effects it is still true as Fenn stated in 1940 that "careful distinction has not always been made between effects on contractility and effects on excitability . . ." In the following discussion of our own experiments we will attempt to make such a distinction, and we will indicate some possibilities regarding the relation between excitation and contraction.

Since the structures for excitation are associated with the membrane while those for contraction are within the fiber, it is evident that the determination of whether the effects of K acting on muscle are due to excitability or contractility changes demands that we know whether or not it has penetrated into the muscle fibers. The muscle membrane is permeable to K, but it is known that K does not begin to penetrate into the cells unless its external concentration is raised above the maintenance level, i.e., the concentration at which K neither enters nor leaves the fibers. Various workers (Fenn and Cobb, '34; Hegnauer, Fenn and Cobb, '34; Boyle and Conway, '41) studying sartorii from different species of frogs, under different conditions of temperature, pH, etc.,

have determined values of the maintenance concentration ranging from 19 to 55 mg %. The experimental conditions of Hegnauer et al. (R. pipiens sartorii, pH 7.2, 22°C., 5 hours' immersion) are closest to ours, and we are therefore adopting their maintenance concentration of 30 mg % for the analysis of our problem.

We must now take into account that the diffusion of KCl into the muscle extracellular space must first occur before there can be any penetration into the fibers. In another paper (Sandow and Mandel, '51) a method based on the work of Hill ('28) has been presented which enables us to determine the effective average concentration of the K ion in the extracellular space as a function of the time after immersion of the muscle in each of our experimental solutions. In the following, all concentrations of KCl will be given in units of equivalent mg % K. Let C_i = the initial concentration of K in the extracellular space of the Ringer's equilibrated muscle (7.8 mg %), and C = the constant, external concentration of K in a test medium. Then, $C_o = C\text{-}C_i$ is the concentration difference between the outside and inside of the muscle determining the diffusion of KCl into the interstitial space. (See Jacobs, '35, for justification of this assumption in respect to the analysis which follows.) At any time, t, following immersion of a muscle in its K-enriched medium, a certain amount of KCl will have diffused into the extracellular space so as to raise the average concentration of K by the increment $\bar{C}$ above that initially present. The value of $\bar{C}$ is found by using the relevant curve of fig. 5 of Hill's paper, which gives $\bar{C}/C_o$, the average degree of saturation, as a function of kt/b^2, in which k = the diffusion constant of KCl into the muscle's extracellular space, and 2b = the muscle's thickness. Here, as in the previous paper (Sandow and Mandel, '51) we take $k = 10^{-4}cm^2/min.$, and 2b = 0.07 cm, and t is expressed in minutes. Now, for any given value of C, having determined $\bar{C}$ at any time we add to this C_i and thus find the effective average concentration of K within the extracellular space at

this time. The results of such calculations are plotted in figure 4.

It is not necessary to give the curves for all our media containing below-maintenance concentrations, since it is obvious that at no time can the K increase in the interstitial space to be able to penetrate. Since no penetration of K into the fibers occurs, all the associated changes in R and T are attributable to the direct action of the extra K only on the fiber

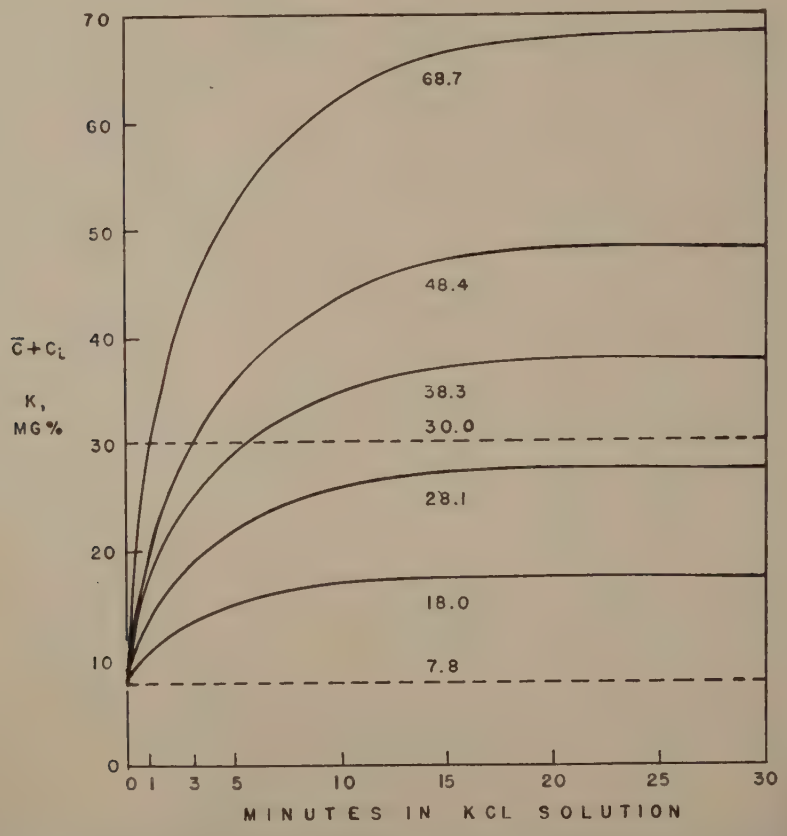

Fig. 4 Curves showing, as a function of time, the rise of the average degree of K saturation in the extracellular space of muscles in K enhanced solutions. The numbers labelling the curves give the constant concentration of K in mg % of each of the solutions. The broken line marked 7.8 shows the initial level of K content in the extracellular space; the broken line at the 30.0 mg % level indicates the maintenance concentration of K.

membrane. The more or less gradual, initial increases in R and T are evidently dependent on the progressive rise of the amount of KCl (i.e., K^+) in the extracellular space, for about 20 minutes is required for this space to become fully saturated in respect to the external KCl. The later diminutions in R and T must then be due to some effect of the prolonged time of action of the K on the fiber membranes. It is thus evident that the presence of extra K in non-penetrating concentrations in the interstitial space can directly affect the surface membrane of the fibers, so that, acting for a short time, there results indirectly determined enhanced outputs of the intrafibril mechanical system which are reversed, though not too drastically, as the increased K continues to act for much longer times.

The especially noteworthy features of the state of the muscles in the media containing K in the above-maintenance range of concentrations is that, depending on the medium, periods of from about 1.8 to 10 minutes are required before the average concentration of KCl in the extracellular space is sufficient to exceed the maintenance level and thus make possible intrafibrillar penetration of K. To appreciate the significance of this, note that R has risen to a maximum at 4 minutes in the 38.3 mg % K medium, at three minutes in the 48.4 mg %, and at one minute in the 68.7 mg %; and at these times the effective average concentrations of K in the interstitial spaces of the respective muscles are 27.3, 30.5, and 27.9 mg %, which are all just at or slightly below the maintenance level. Somewhat similar relations also hold for the immediate augmentations in T. Thus, even for the muscles tested in the set of media having higher K concentrations, the initial rises in R and T are indirect consequences of the action of K at only the fiber surfaces. Very soon after these initial effects have been completed, and R and T are rapidly falling, sufficient diffusion of KCl has occurred to raise the interstitial K concentration to penetration levels. Hence these later effects may be associated with the entrance of K into the fibers. The validity of this inference is indicated in figures 1 and 2 by the

fact that on the average the rate of fall of both R and T in these media is about 14 times as great as it is in the below-maintenance solutions. It is reasonable to infer that this quantitatively distinct behavior is due to the penetration of K, and therefore to the direct action of this substance on the contractile system.

But this inference requires some qualification in view of our findings that the addition of KNO_3 or K_2SO_4 to Ringer's solution so as to raise the K concentration to 48.4 mg %, leads to altered mechanical behavior indistinguishable from that due to KCl. For, according to Conway and Moore ('45), the rate of penetration of KNO_3 into muscle cells is only about one-half of that of KCl, while K_2SO_4 does not penetrate at all, these differences being attributed to corresponding reductions in permeability to the associated anions. It would seem as though these permeability differences would be reflected in corresponding alterations in the time course and magnitude of our K effects. But our results prove that they are not. Therefore, we infer that the K of KNO_3 and even of K_2SO_4 enters the fibers by exchange with some intracellular cation (e.g., H^+ or Na^+), or by association with the chloride of the other components of the medium, and thus the K behaves just as it would had it been added as KCl.

Although the changes in the mechanical variables as a function of external K concentration show correlation with the possibility of K penetration, the alterations of the action potential present no such correlation for these involve only decreases in all our experimental media. Figure 3 demonstrates, however, that increasing the K up to 23.0 mg % causes very little, if any, increase in rate with which the action potential diminishes; but a sharp increase occurs in the medium containing 28.1 mg % K which is still just below maintenance level. This difference in dependence of the mechanical and electrical systems on K penetration might be expected, for the action potential is a membrane response and thus it would be more sensitively affected by the K acting only at the fiber surface. Nevertheless, it is noteworthy that the amount of

K acting at the surface must exceed a certain rather critical level which is about three times the normal in order to cause a marked deterioration of the action potential mechanism. Essentially comparable results have been obtained by many other workers, especially following the finding of Duliere and Horton ('29) that muscle soon becomes inexcitable in a Ringer's solution containing three to 4 times the normal K.

It is now of interest to discuss some implications of the result of our diffusion analysis which indicate that K acting only at the fiber membrane causes a depression of the action potential and yet a potentiation of the mechanical responses. In this connection, it is important to note that our discussion of the relation between the initial changes in excitability and contractility proves that any modifications we have observed on the whole muscle are actually due to equivalent alterations in each of the component fibers. Thus our problem reduces to that of determining the mechanism by which an action of K on the membrane can lead to a change in the mechanical behavior of the intrafibrillar contractile system.

Our view is that K influences two distinct membrane structures. One of these is the system responsible for the electrical behavior which is well known not only as a membrane function, but also as one whose expression in the action potential is easily depressed by excess K. The other system must then determine events which pass inward and thus lead to the activation of the contractile material; and this we infer to be distinct from the electrical system since K, contrary to its effect on the action potential, can enhance the mechanical output presumably by its special action on this part of the membrane and its associated inward moving process.

The full discussion of these relations in the coupling of excitation to contraction is beyond the province of this paper, for it requires a detailed analysis of the kinetics of excitation and of activation of contraction, and a thorough study of the special mechanism of excitation under massive stimulation. Such discussion will therefore be presented elsewhere (see, e.g., Sandow and Kahn, '52). But of relevance here is the

fact that our results demonstrating a negative correlation between action potential and mechanical changes support the frequently expressed view (e.g., Rosenblueth, '50) that the action potential serves as a trigger in the initiation of excitation-contraction coupling. Some pertinent data are worth mentioning. Thus, after our 48.4 mg % K solution has acted for 5 minutes, the action potential is only 65% of normal but the R and T outputs are respectively 260% and 135%. And even later when the mechanical responses are beginning to decrease, a similar disparity exists, as shown, e.g., after 30 minutes in the 28.1 mg % K medium when A is down to 20% of normal and the R and T values are respectively 140% and 112%. These results prove that the spike operates in excitation-contraction coupling with a quite large safety factor, and it is in this sense that we attribute to it the function of a trigger mechanism. Triggers of this general type are already known in connection, e.g., with the roles of the spike potential and of the end-plate potential in their functions determining, respectively, fiber conduction and neuromuscular transmission. In these cases, however, the trigger acts to generate a response similar to itself, i.e., another membrane depolarization. But in the case of coupling of excitation to contraction, our analysis suggests that the spike potential triggers the setting-off of the special membrane response which we have inferred moves inward and then serves to activate the contractile system.

In searching for an explanation of the way in which K modifies the membrane responses, we may rule out known effects of K on resting muscle metabolism. Thus Hegnauer and coworkers ('34) found that the various metabolic changes they studied did not appear until the external K concentration was raised to at least 25–30 mg %, yet our results show very pronounced changes in R and T when the K concentration is no more than 18.0 mg %. Also, the heat changes of resting K-treated muscles (Solandt, '36; Smith and Solandt, '38) cannot be correlated with our observations, for they were not very pronounced unless the K was above 7 × normal, i.e.,

above the highest concentration we investigated. And in the 7 × K medium the increase in heat output required an hour to achieve maximum and it then decreased during the next 4 hours — thus showing a kinetics quite unlike that we note for R, T, and A in our quite comparable medium containing 68.7 mg % K. Since these metabolic and heat modifications are most likely due to alterations of the great mass of the intrafibrillar material of the muscle, their elimination as the source of our K-induced changes, all the more establishes these changes as primarily membrane effects.

One possibility of a surface change is the depolarization due to the K. But Mandel ('51) studied the kinetics of the depolarization of muscle caused by our experimental solutions and found (see also, Sandow and Mandel, '51) that the depolarization, for each medium, developed roughly as a logarithmic function of time, attaining a steady maximum value in about 15 minutes. Since the kinetics of the depolarization is entirely different from that of the simultaneously occurring initial modifications in either R or T, it is evident that changes in resting polarization may be eliminated as a factor in attempting to account for the mechanical changes of interest.

It may be significant that various cells, e.g., the fertilized Arbacia egg, internally release free Ca ion from their cortical regions when treated externally with excess K (Churnev and Moser, '40). Although it is not known whether a similar process occurs in K-treated muscle, Ca is released from normal muscle upon electric stimulation (Woodward, '49). The K-induced liberation of Ca was obtained in cells treated with much higher K concentration than we used, so that our experimental muscles at rest may not have had any appreciable release of Ca, but rather a "loosening" of the membrane which sensitized it so that excitation caused a greater than normal Ca-release. These considerations thus suggest that the K-sensitive membrane structure we have inferred in addition to that determining the electrical behavior may be some Ca-complex, and that the inward moving process we postulate as a link between membrane processes and activation of the con-

tractile system is at least initiated by the liberation of free Ca from this complex.

We now briefly discuss the secondary, depressive effects of K which follow the initial enhancements of R and T. These fall into two groups: (1) those obtained in the media containing up to 28.1 mg % K which involve only moderate decreases in the mechanical responses, and (2) those in the media of higher K concentration which exhibit very rapid and profound decreases in these outputs. Since the changes of the first group occur evidently in the absence of K penetration, it seems that mere prolongation of the influence of K on the membrane results in a gradual reversal of the full augmentative effects engendered during the first few minutes of action of this substance, probably by increasing to an injurious level the previously postulated "loosening" of the membrane.

The second group of effects is associated with penetration of K so that now both the membrane and inner protoplasm of the fibers are affected by this agent. Whatever may be the actual state of affairs when muscles are exposed to the media containing K in above maintenance concentration, it is evident that two opposing types of K effects will now develop: the initial, mechanically augmentative one, followed soon by the depressive one. The relative intensities of these two actions as a function of time will depend on the external K concentration. In this general way we may account for the fact that the initial rises of R and of T in our 68.7 mg % medium do not attain maxima at all as high as they are in, e.g., 48.4 mg % K; for with the higher concentration penetration is so much more rapid that the depressive effects appear very quickly and thus prevent full expression of the initial, purely surface mediated augmentations.

It is difficult to assess the mechanism of the K actions resulting from penetration of K. Heilbrunn and Wiercinzki ('47) have shown that injection of K into the interior of the muscle fiber has no discernible mechanical effect. But such treated fibers were studied at rest; if they were stimulated the results might be otherwise. K is known to have a great

variety of effects on both the physical state of the muscle contractile protein, actomyosin, and on the enzymatic activity of myosin-ATPase when these systems are studied in variously extracted forms (see, e.g., Singher and Meister, '45; Szent-Györgyi, '48; Mommaerts, '50; Perry, '51). Probably of greatest interest in relation to the explanation of our observed depressive K actions, are Perry's experiments which demonstrated that the ATPase activity of myofibrils, extracted from muscle by relatively mild procedures, was rather sharply depressed by K concentrations just above the content found in the intact fiber. In view of the generally current assumption that ATPase activity is somehow involved in the mechanical response of muscle, this may indicate that the inhibition of this enzymatic activity by K is responsible for the lowered mechanical responses of the muscles of our experiments.

SUMMARY

1. Studies have been made of the influence of enhanced concentrations of K, from 18.0 to 68.7 mg %, on the latent period changes, twitch tension, and action potential of isometric contractions of frog sartorius muscles excited by both massive and wire electrode methods. The alterations of the various responses have been determined throughout a total two-hour period of action of K, with special attention being given to the generally quite rapid changes that appear within the first few minutes.

2. In all K media, the mechanical parameters, depth of latency relaxation (R) and twitch tension (T), first increase with time and then decrease. These modifications, especially of R, are much more rapidly effected, involve generally greater initial increases and much more profound subsequent decreases in a higher range of K concentrations, at and above 38.3 mg %, than in a lower range up to 28.1 mg %.

3. The action potential spike (A) decreases progressively with time in all K solutions, and the rate of decrease is much greater in K concentrations at and above 28.1 mg % than it is in lower concentrations.

4. An anaylsis of the diffusion of K into the extracellular muscle space as against K penetration into the fibers, shows that (a) *all* the observed mechanical changes in media having up to 28.1 mg % K, (b) only the *initial* increases in R and T in media of 38.3 mg % and greater concentration, and (c) the decreases in A in media up to 28.1 mg % are involved with diffusion of the extra K only into the extracellular space. These various effects are therefore attributed to a direct action of K on the fiber surface which results in consequent indirect effects on the mechanical behavior of the intracellular contractile material. The sharp decreases in R and T which follow the initial increases mentioned in (b) above, and in A in K solutions higher than 28.1 mg %, are associated with penetration of K into the fibers, and thus here the K may influence the various muscle responses by a direct action on the intracellular contractile material and by a more thorough saturation of the membrane material.

5. Since K, acting only at the fiber membrane, causes alterations in mechanical, as well as electrical, responses, it is inferred that the K affects two membrane systems: one concerned with the electrical behavior, and the other involved in linking excitation to the activation of the contractile system.

6. The generation of full and even potentiated R and T outputs in association with greatly decreased values of A signifies that the action potential serves as a trigger, having a large safety factor, in the initiation of the coupling of excitation to contraction.

LITERATURE CITED

BAETJER, A. M. 1935 The effect of potassium (and calcium) on the contractions of mammalian skeletal muscle. Am. J. Physiol., *112:* 147–151.

BOYLE, P. J., AND E. J. CONWAY 1941 Potassium accumulation in muscle and associated changes. J. Physiol., *100:* 1–63.

BROWN, D. E. S. 1936 The effect of rapid compression upon events in the isometric contraction of skeletal muscle. J. Cell. and Comp. Physiol., *8:* 141–157.

————1941 The regulation of energy exchange in contracting muscle. Biol. Symp., *3:* 161–190.

BROWN, D. E. S., AND F. J. M. SICHEL 1936 The isometric contraction of isolated muscle fibers. J. Cell. and Comp. Physiol., *8:* 315–328.

BROWN, G. L. 1937 The action of potassium chloride on mammalian muscle. J. Physiol., *91:* 4–5 P.

BROWN, G. L., AND U. S. VON EULER 1938 The after effects of tetanus on mammalian muscles. J. Physiol., *93:* 39–60.

CARLETON, B. H., H. A. BLAIR AND W. B. LATCHFORD 1938 The effects of the chlorides of potassium, calcium and sodium on the alpha excitability of muscle. J. Cell. and Comp. Physiol., *12:* 223–236.

CHAO, I. 1937 Threshold intensity and height of submaximal contraction as criteria of muscular excitability — illustrated by the action of ions. The Science Reports of National Tsing Hua University, Series B, *2:* 183–190.

CHURNEY, L., AND F. MOSER 1940 The effects of excess potassium on the cortex of the fertilised egg of Arbacia punctulata. Physiol. Zool., *13:* 212–218.

CONWAY, E. J., AND P. T. MOORE 1945 Cation and anion permeability constants for the muscle fibre membrane. Nature, *156:* 170–172.

DULIERE, W., AND H. V. HORTON 1929 The reversible loss of excitability in isolated amphibian voluntary muscle. J. Physiol., *67:* 152–165.

FENN, W. O. 1940 The role of potassium in physiological processes. Physiol. Rev., *20:* 377–415.

FENN, W. O., AND D. M. COBB 1934 The potassium equilibrium in muscle. J. Gen. Physiol., *17:* 629–656.

GUTTMAN, S. A., AND MCK. CATTELL 1940 The effects of digitoxin and potassium on striated muscle. J. Pharmacol. and Exp. Therap., *68:* 267–277.

HEGNAUER, A. H., W. O. FENN AND D. M. COBB 1934 The cause of the rise of oxygen consumption of frog muscles in excess potassium. J. Cell. and Comp. Physiol., *4:* 505–526.

HEILBRUNN, L. V., AND F. J. WIERCINZKI 1947 The action of various cations on muscle protoplasm. J. Cell. and Comp. Physiol., *29:* 15–32.

HILL, A. V. 1928 The diffusion of oxygen and lactic acid through tissues. Proc. Roy. Soc. B, *104:* 39–96.

HÖBER, R. 1945 Physical Chemistry of Cells and Tissues. Blakiston, Philadelphia.

HODGKIN, A. L., AND B. KATZ 1949 The effect of sodium ions on the electrical activity of the giant axon of the squid. J. Physiol., *108:* 37–77.

JACOBS, M. H. 1935 Diffusion processes. Ergeb. der Biol., *12:* 1–160.

KAHN, A. J., AND A. SANDOW 1950 The potentiation of muscular contraction by the nitrate-ion. Science, *112:* 647–649.

MANDEL, H. 1951 Master's Thesis, Department of Biology, Washington Square College of Arts and Science, New York University.

MOMMAERTS, W. F. H. M. 1950 Muscular contraction — a topic in molecular physiology. Interscience Publishers, New York.

PERRY, S. V. 1951 The adenosinetriphosphatase activity of myofibrils isolated from skeletal muscle. Biochem. J., *48:* 257–265.

ROSENBLUETH, A. 1950 The transmission of nerve impulses at neuroeffector junctions and peripheral synapses. John Wiley and Sons, Inc., New York.

SANDOW, A. 1944 Studies on the latent period of muscular contraction. Method. General properties of latency relaxation. J. Cell. and Comp. Physiol., *24:* 221–256.

SANDOW, A. 1947 Latency relaxation and a theory of muscular mechano-chemical coupling. Ann. N. Y. Acad. Sci., *47:* 895–929.

———— 1948 Transverse latency relaxations of muscle stimulated by massive transverse shocks. Fed. Proc., 7, no. 1: 107–108.

SANDOW, A., AND A. J. KAHN 1952 Membrane processes in excitation-contraction coupling. Fed. Proc., 11, no. 1, 136.

SANDOW, A., AND A. G. KARCZMAR 1950 Effect of iodoacetate on changes in muscular latency induced by activity. Am. J. Physiol., *163:* 247–259.

SANDOW, A., AND H. MANDEL 1951 Effects of potassium and rubidium on the resting potential of muscle. J. Cell. and Comp. Physiol., *38:* 271–291.

SINGHER, H. O., AND A. MEISTER 1945 The adenosinetriphosphatase activity of myosin preparations. J. Biol. Chem., *159:* 491–501.

SOLANDT, D. Y. 1936 The effect of potassium on the excitability and resting metabolism of frog's muscle. J. Physiol., *86:* 162–170.

SMITH, C. G., AND D. Y. SOLANDT 1938 The relation of contracture to the increment in the resting heat production of muscle under the influence of potassium. J. Physiol., *93:* 305–311.

SZENT-GYÖRGYI, A. 1948 Nature of life — a study on muscle. Academic Press, New York.

WALKER, S. M. 1947a The response of the triceps surae of normal, adrenalectomized, desoxycorticosterone acetate-treated and KCl-treated rats to direct and indirect, single and repetitive stimulation. Am. J. Physiol., *149:* 7–23.

———— 1947b Observations on the action potentials induced by indirect stimulation of skeletal muscle in desoxycorticosterone acetate-treated rats on a low potassium diet. Am. J. Physiol., *150:* 451–460.

WOODWARD, A. A., JR. 1949 The release of radioactive Ca^{45} from muscle during contraction. Biol. Bull., *97:* 264.

CYTOLOGICAL EFFECTS OF X-IRRADIATION AND COLCHICINE APPLIED SEPARATELY AND IN COMBINATION [1]

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THREE FIGURES

In previous papers (Allen, Schjeide, and Hochwald, '50, '51; Allen, Schjeide, and Piccirillo, '51) it has been shown that 500 r of x-irradiation causes hematopoietic cells of the tadpole to break down in a characteristic manner as they approach mitosis. It also has been shown that, under similar conditions, application of 10 γ of colchicine causes all dividing cells in these tissues to be arrested at late prophase (Allen, Schjeide, and Piccirillo, '51; Schjeide and Allen, '51). Tissues treated with a combination of these two agents display a type of damage which is mainly characteristic of x-irradiation (Schjeide and Allen, '51). Thus the actual kinds of change produced by these three types of treatment show differences of varying degree.

Yet, when treatments consisting of 500 r of x-irradiation, 10 γ of colchicine, and 500 r of x-irradiation in combination with 10 γ colchicine, were administered to three separate groups of tadpoles, almost *identical percentages of total cell alteration were observed at 24 hours in all three groups* (Schjeide and Allen, '51).

In the present paper we are especially concerned with one aspect of the above findings, namely that the combination of x-irradiation and colchicine produces no more cell altera-

[1] This paper is based on work performed under Contract No. AT-04-1-GEN-12 between the Atomic Energy Commission and the University of California at Los Angeles.

tion at the end of a 24 hour interval than does either agent alone. We ask the specific question, "Does this relationship exist at other time intervals?"

It should be pointed out here that *two* factors operate to give the observed percentage of cell alteration at this time: (1) the rate of appearance of damage in cells as they reach a critical phase or condition, and (2) the rate of dissolution and consequent disappearance of damaged cells. The changes which we see at the end of 24 hours are a result of the interaction of these two factors.

Thus we have no quarantee that the relationships in amounts of destruction as observed at 24 hours post-treatment are necessarily identical with those observed at other intervals of time.

In the present work we have investigated the percentages of cell alteration which occur at 4, 8, 12, 16, 20 and 24 hours. X-irradiation and colchicine were applied separately and in combination.

This information is of special interest in view of the present controversy regarding the combined application of colchicine and x-irradiation for the destruction or inhibition of abnormal growth (Levine).

MATERIALS AND METHODS

1. Tadpoles of Rana catesbiana were divided into three like groups (80 to 100 mm length; 10 to 15 gm weight). The tadpoles used for the experiment in figure 1 were of a different source than those used for the experiment in figure 2.

2. These groups were treated as follows:

Group A. Five hundred r of x-irradiation only (x-irradiation treatment was given with a Picker Industrial Unit). The x-irradiation factors were, 250 kv, 15 Ma, 35 cm T.O.D., 0.21 mm Cu inherent plus 0.5 mm parabolic Cu, plus 1 mm Al filters, H.V.L. = 1.9 mm Cu center of parabolic filter. Roentgens measured in air.

Group B. Ten gamma of colchicine injected into the tail (0.65% saline solution used as vehicle).

Group C. Ten gamma of colchicine followed immediately by 500 r of x-irradiation.

3. The tadpoles were kept for 4, 8, 12, 16, 20 and 24 hours post-treatment at 18°C. and at 20°C., and were then killed.

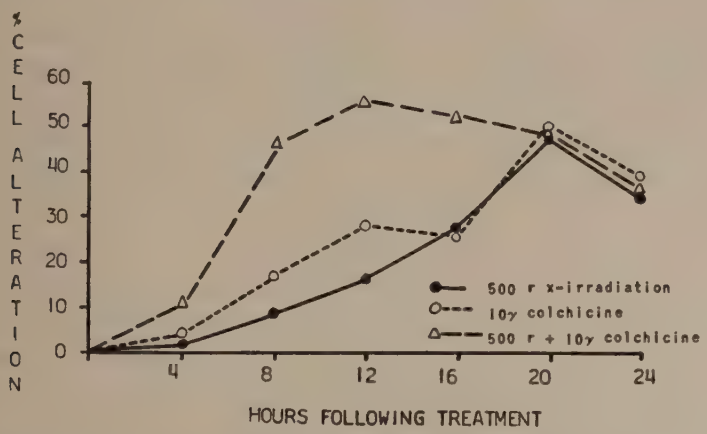

Fig. 1 The amounts of cell alteration observed at various intervals after treatment in the hematopoietic cells of tadpoles (Rana catesbiana). Temperature 18°C. A total of 90 tadpoles.

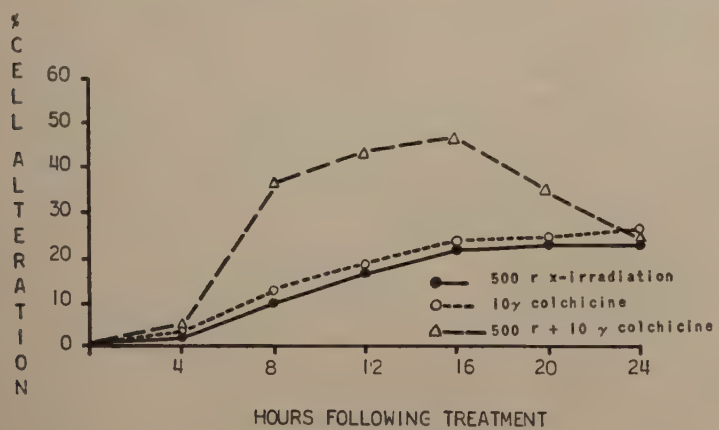

Fig. 2 The amounts of cell alteration observed at various intervals post-treatment in the hematopoietic cells of tadpoles (Rana catesbiana). Temperature 20°C. A total of 119 tadpoles. These tadpoles were obtained from a different source than those in figure 1.

4. After killing, the kidneys, which contain a large portion of the hematopoietic cells in these amphibians, were immediately excised and fixed in Bouin's fluid. These were later stained with standard blood stains.

5. On the stained material, the percentage of destruction and/or division observable in the tissue was determined by tallying the damaged or mitotic versus the non-damaged and non-mitotic cells within 4 microscopic fields (each defined by a Whipple Grid).

6. Comparison was then made between the three groups of experimental animals as sacrificed at the various intervals.

RESULTS

The results are summarized graphically in figures 1 and 2. It was seen that, after a 4 hour period which featured only small differences, the observed amounts of cell alteration in the hematopoietic tissues became characteristically different for each of the three types of treatment. The combination of colchicine and x-irradiation showed a particularly large rise in the amount of destruction between 4 and 8 hours post-treatment.[2]

In the case of 500 r of x-irradiation alone, it is seen in both figure 1 and figure 2 that following the aforementioned 4 hour lag, the apparent percentage of destruction rose linearly to reach a peak of 20 hours. However, from 20 hours to 24 hours a definite decline was observed in the percentage of destroyed cells in the first experiment (fig. 1) and a plateau was seen in a second experiment (fig. 2). The decline in numbers of destroyed cells during this latter interval (fig. 1) is evidence that damaged cells were being removed from the scene at a rate which modified the overall picture of cell

[2] It should be pointed out that the changes in amount of observed cell alteration up to at least 8 hours post-treatment provide a true indication of rate of cell alteration. After 8 hours and especially by 12 and 16 hours the changes in observed amounts of alteration do not give a true picture of the *rate* of cell alteration. This is due to the introduction of a second process in addition to cell alteration, namely the disappearance of damaged cells.

destruction. Cytological evidence of cell disappearance first appeared at about 12 hours. This was evident by the presence of hollow spaces and aggregated nuclear debris scattered throughout the compact tissue. Criteria for damage by x-irradiation consisted of any change in staining or shape of the normal nucleus.

When 10 γ of colchicine was injected into the tails of tadpoles it was found that the colchicine moderately increased the percentage of cell alteration in the hematopoietic cells at 8 and 12 hours over that obtained with x-irradiation. This difference was significant ($P = .05$) in the first experiment (fig. 1) but was not significant in the second experiment (fig. 2). One might expect such a difference on the basis of temporary inhibition of division in the irradiated tissue. It can be seen in figure 1 that the numbers of altered cells in the cases of 500 r and 10 γ of colchicine were almost identical at 4, 16, 20 and 24 hours. Cytologically, criteria for the effect of colchicine were arrest of mitosis in the late prophase and the various stages of destruction which followed arrest. It also appeared in some cases that the colchicine treatment had slightly altered the capacity of the cells to take up stain. This was especially true of the cytoplasm. But such alteration was not used as a criterion of cellular damage.

When 10 γ of colchicine was followed immediately by 500 r x-irradiation a strikingly different amount of cell alteration resulted. It was observed that the amount of cell destruction at especially 8, 12 and 16 hours far exceeded the amount of arrested mitoses in the cases where colchicine was employed alone or destruction where 500 r of x-irradiation was the sole treatment. In fact, *at 8 and 12 hours the destruction was more than double that obtained by the use of either agent alone.*

Since amounts of observed destruction provide a valid basis for calculations of rate up until the beginning of cell disappearance (8–12 hours), it can also be said that the *rate* of destruction seemed to be especially accelerated between 4 and 8 hours after initiating the combined treatment. After

20 to 24 hours, removal of damaged cells and changing rates of destruction had taken place to the extent that tadpoles treated with colchicine plus x-irradiation showed a nearly identical percentage of observed cell alteration to those treated with x-irradiation alone or colchicine alone.

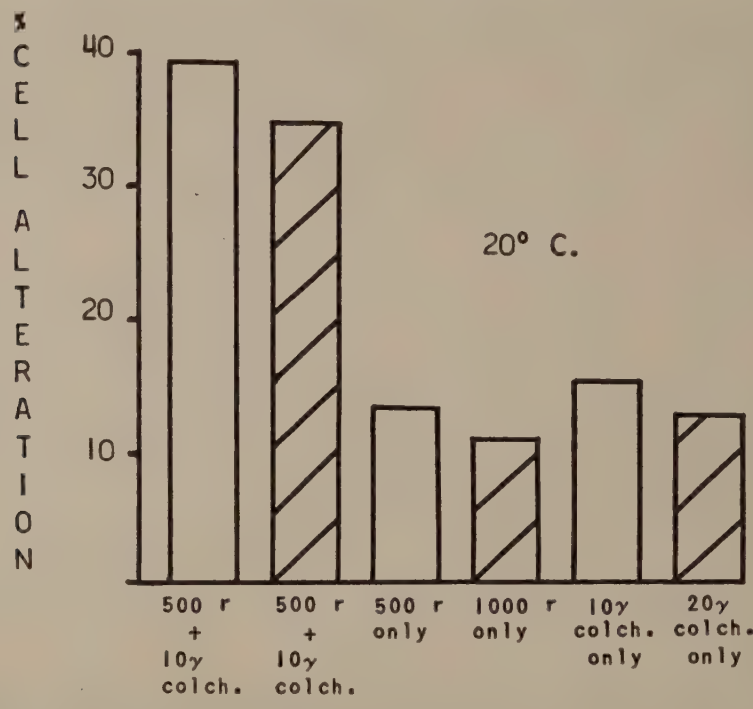

Fig. 3 Percentage of cell alteration observed after different doses of x-irradiation and colchicine applied separately and together. The plain columns represent the percentage of destruction observed with standard dosages (500 r x-irradiation and 10 γ colchicine). The lined columns show the results of a subsequent experiment in which the doses of irradiation alone and colchicine alone were doubled and the combined dosage was standard. All tadpoles were maintained at 20°C. and killed 10 hours post-treatment.

Since it seemed possible that the exaggerated amount of destruction occurring as a result of the combined use of colchicine and x-irradiation might be due merely to a non-specific doubling of dosage, an experiment was performed in which double doses of each agent were used. It was seen

(fig. 3) that the use of 20 γ of colchicine rather than 10 γ of colchicine and 1000 r of irradiation in place of 500 r of x-irradiation not only failed to produce the exaggerated effect in the combination of the lesser amounts of the two agents, but also failed to increase the cell alteration over that obtained with 500 r alone or 10 γ of colchicine alone. It was further observed that hematopoietic tissue exposed to only 5 γ colchicine displayed more cells obviously arrested in late prophase as opposed to tissues receiving 10 γ and 20 γ of colchicine although the sum of the obviously mitotic cells and degenerating cells was the same as in these higher dosage groups. A dose of only 1 γ colchicine produced mitotic arrest in the metaphase rather than the prophase. There were fewer degenerating cells in this group but cells were observed which had escaped colchicine arrest.

An approximation of the rate of mitosis in these two groups of tadpoles may be obtained by calculations based on the amount of colchicine arrest occurring between 4 and 12 hours. This interval seems to be free from other influences such as lag and rate of cell removal. In the first group of tadpoles the rate of mitosis in each 100 cells was three per hour. In the second group the rate was about two per hour.

DISCUSSION

One of the most significant features of the results obtained by the combined use of colchicine and x-irradiation is the early acceleration in rate of cell breakdown over that obtained with x-irradiation alone or colchicine alone.

It might seem that an obvious explanation for the increased rate of cell alteration in this case would be that x-irradiation affects the cell in one finite stage and colchinine affects the cell at another stage. Thus the two agents applied together would produce damage within a given time interval ranging from the same to double that by either agent alone, depending on the distance between the two sensitive stages.[3] However we

[3] Actually the increase in rate of destruction with the combination of the two agents is much more than double during the period 4–8 hours.

have already shown (Schjeide and Allen, '51) that the normal sensitive stages, when each agent is used alone, are quite close together. Cells exposed to 500 r irradiation break down only when they reach early prophase. Arrest by 10 γ colchicine is observed only when the cell enters late prophase (Schjeide and Allen, '51). The per cent of the total cellular cycle which spans early prophase to late prophase is negligible. Thus there would theoretically be a negligible increase in damage due to these causes. Actually, when tissue treated with 500 r x-irradiation alone was examined, no dividing cells were seen. This indicates that 500 r x-irradiation probably destroys cells which are in any stage of mitosis at the time of treatment. But since these are so few in number no increase in destruction is noticeable.

It is possible that the combination of these agents serves as a stimulus for increased cell division and consequent increased destruction. However, the evidence is against the notion that colchicine alone is capable of stimulating division (Levine), or that irradiation alone can stimulate cell division (Giese, '47).

Whatever the nature of this initial wave of destruction where the two agents have been applied simultaneously, equivalent *amounts* of cell alteration are present in all tissues between 20 and 24 hours (figs. 1 and 2). It would appear that equivalent *rates* of cell alteration are indicated by this close agreement in amount of observed cell alteration. Although this may be true, it should again be emphasized that amounts of cell alteration observed after 12 hours may be misleading since the rate of cell removal has by this time become superimposed upon the rate of cell destruction.

With these characteristic features of our system in mind and the interpretations which we have accorded them, we suggest the following mechanisms of cell breakdown in those cases where x-irradiation and colchicine are applied simultaneously.

Mechanism of destruction. One hypothesis which may explain the great initial increase in rate of destruction when

500 r and colchicine have been administered together is that the products of irradiation influence or are influenced by the colchicine so as to create much more toxic products. Such products might act in a destructive way at different stages than those recognized as being susceptible to either agent alone. In particular the products of stress induced by x-irradiation may act synergically with colchicine. The well known action of very small amounts of colchicine in alleviating conditions of gout (which are accompanied by a fall in certain of the adrenal hormones) might have a definite bearing in the present case. Indeed, a few of our tadpoles have shown a surprising amount of cellular destruction when injected with 10 γ of colchicine alone. There is reason to believe that these tadpoles had been subjected to conditions of stress. Actually we have no evidence as to whether colchicine and x-irradiation applied together will increase the amount of destruction in tissues if these are both confined to a localized part of the body.

A second hypothesis assumes a relationship between those agents which are affected by colchicine and those which are responsive to x-irradiation. For example: suppose some cellular factor (A) in the cell is susceptible to irradiation. Assume that this factor is necessary for the elaboration of a second agent which is critical during the interkinetic phase. Suppose further that a second cellular factor (B) in the cell is relatively more sensitive to colchicine than to irradiation and that this factor is necessary for the elaboration of the same agent (X) as is elaborated by the x-irradiation sensitive factor. Thus, either "A" or "B" can supply "X" but when both factors are destroyed simultaneously so is factor "X." A temporary wave of destruction is the result. The extent of this wave would be a function of the extent of the cellular cycle which is dependent on the intact factor "X."

Other investigations. The application of colchicine in combination with x-irradiation has been investigated by workers in the field of cancer destruction and inhibition. The results,

which have been largely inconclusive, have been given an excellent review by Levine. Most of the previous work has been carried out with a significantly different technique than that which we have employed. The colchicine is most of these cases has been applied sufficiently in advance of x-irradiation (8 to 48 hours) so that many of the cells are held in mitosis (Levine). In our work the colchicine injections were followed immediately by 500 r x-irradiation.

Previous conclusions. The generally close correspondence of amount of cell alteration produced at these intervals by colchicine and x-irradiation (figs. 1 and 2) lends further support to our previous concept (Schjeide and Allen, '51) that 500 r x-irradiation affects normal hematopoietic cells by causing them to break down at or near mitosis. That no whole mitotic figures are observed in irradiated tissue at any of the intervals tested lends additional confirmation to this concept.[4]

SUMMARY

The combined application of 500 r x-irradiation and 10 γ colchicine produced an early aceleration in the rate of breakdown of the hematopoietic cells in tadpoles (Rana catesbiana) as compared to the cell alterations produced by 500 r x-irradiation alone or 10 γ colchicine alone.

This acceleration occurred between 4 and 8 hours post-treatment. A lag was evident in all three groups up to about 4 hours. (The tadpoles were kept at 18° to 20°C.)

No such acceleration was observed when double amounts of either agent alone were administered. In fact, doubling

[4] Work subsequent to that above has demonstrated that as little as 100 r of irradiation plus 10 γ of colchicine will produce an accelerated breakdown of hematopoietic cells just as does 500 r plus 10 γ of colchicine. Likewise 500 r plus as little as 1 γ of colchicine produces the accelerated damage. But 100r plus 1 γ of colchicine gives no more destruction than does 1 γ of colchicine alone (approximately 8% at 12 hours post treatment). (One hundred r of x-irradiation produces about 3% destruction in 12 hours.) The acceleration of damage apparently takes place only at and above 13°C. and anoxia will abolish the effect. The experiments must be strictly controlled as certain yet undefined factors sometimes cause tadpoles treated with colchicine *only* to show the accelerated rate of cell breakdown which is characteristically associated with the combination of the two agents.

the dosage had no detectable effect in increasing the amount of cell alteration.

At 8, 12 and 16 hours post-treatment the observed amount (not rate) of hematopoietic cell destruction in the case of 10 γ colchicine plus 500 r x-irradiation was *more* than double the amount of cell alteration observed in the case of colchicine alone or x-irradiation alone.

The amounts of cell alteration in the case of (a) colchicine and x-rays in combination, (b) 500 r x-irradiation alone, and (c) 10 γ colchicine alone, became identical at 20 to 24 hours post-treatment.

Possible mechanisms of the peculiar action of colchicine in combination with x-rays were discussed.

LITERATURE CITED

Allen, B. M., O. A. Schjeide and L. B. Hochwald 1950 Relation of low temperatures to x-ray injury of hematopoietic tissue of tadpoles. Soc. for Exp. Biol. and Med., *73:* 60–62.

———— 1951 The influence of temperature upon the destruction of x-rayed hematopoietic cells of tadpoles. J. Cell. and Comp. Physiol., *38:* 69–82.

Allen, B. M., O. A. Schjeide and R. Piccirillo 1951 The influence of anoxia upon destruction and mitosis of the hematopoietic cells of x-irradiated tadpoles. U.C.L.A. Atomic Energy Project Report 149.

Giese, A. C. 1947 Radiations and cell division. The Quarterly Review of Biology, *22:* 253–282.

Levine, M. The action of colchicine on cell division in human cancer, animal and plant tissues. Annals N. Y. Acad. Science, *51:* 1365–1408.

Schjeide, O. A., and B. M. Allen 1951 The relation of mitosis to the manifestation of x-ray damage in hematopoietic cells of tadpoles. J. Cell. and Comp. Physiol., *38:* 51–68.

AN ACTOMYOSIN-LIKE SUBSTANCE FROM THE PLASMODIUM OF A MYXOMYCETE

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FIFTEEN FIGURES

INTRODUCTION

This work is concerned with the study of an extract which, like muscle actomyosin, markedly changes in viscosity in the presence of low concentrations of adenosine triphosphate. This extract which was obtained from the plasmodium of the myxomycete *Physarum polycephalum,* was studied in the hope that it may give insight in the mechanism by which unspecialized or primitive tissue is able to convert chemical energy into mechanical work.

One of the fundamental qualities of living matter is the ability to perform mechanical work. This characteristic has reached its evolutionary culmination in the muscle cell. Indeed the capacity for work in this highly specialized machine is so well developed that one is apt to ignore it in other cells. And yet all cells, at least at some stage of their life history, engage in mechanical work. The beat of the cilium and flagellum, the streaming of cytoplasm, the movement of chromosomes, the movement of amoeboid cells, the gliding of diatoms,

[1] This work was done while holding a Public Health Research Fellowship of the National Institutes of Health. The work was supported in part by grants from The American Cancer Society upon nomination by the Committee on Growth, and from The National Cancer Institute of U. S. Public Health Service. Present address: University Laboratory of Physical Chemistry Related to Medicine and Public Health, Harvard University, Boston, Massachusetts. Address after October 1, 1951: Biochemical Laboratory, Cambridge University, Cambridge, England.

all are examples of the ability of living matter to transform chemical energy into mechanical work.

The basic postulate pervading this work is the assumption that what is true for muscle may also be true for less specialized cells or, to put it into evolutionary phraseology, the highly specialized mechanism in muscle must find its origin in the primitive cell.

The rapid strides of muscle chemistry during the last few years (Szent-Györgyi, '51; Weber and Portzehl, '52) provide this postulate with an operational basis for we may now apply this knowledge to other biological forms of mechanical work.

One of the greatest achievements of muscle chemistry is the isolation of two proteins which seem to retain some of the properties of the muscle cell. A spectacular example of this is the delicate manner in which adenosine triphosphate (ATP), the key energy mediator of cellular metabolism, controls some of the physical properties of these proteins. The two proteins, actin and myosin, are known to form a complex (actomyosin). The addition of ATP to this complex will markedly lower its viscosity when the latter is in solution and will cause it to contract when it is precipitated. It is very tempting to assume that this contraction observed *in vitro* is the basis of the mechanism of muscular motion.

It was thought that a search for a similar protein in undifferentiated tissue might achieve more than the mere extension of existing knowledge to still another tissue, since studies on the undifferentiated cells may reveal aspects of the motile mechanism which the more specialized muscle cell conceals.

MATERIAL AND METHODS

The organism selected for this study is the myxomycete *Physarum polycephalum*. It was chosen because:

1. Its massive plasmodium is engaged in vigorous protoplasmic streaming throughout its vegetative existence. It is

therefore likely to contain relatively large amounts of the morphoplastic [2] system.

2. The plasmodium is naked and multinucleate. The absence of a cell wall or multicellular organization should facilitate the extraction of proteins and permit the utilization of mild methods of tissue fragmentation.

3. The organism can be grown in large quantities and with a minimum of labor. As proof of this, a time sheet was

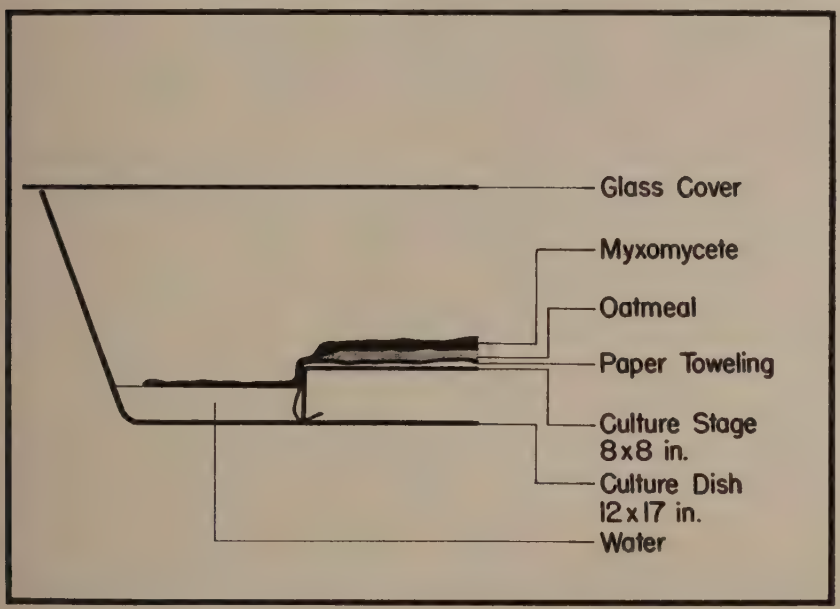

Fig. 1 Cross section view of myxomycete culture.

constructed which indicated that the growth of 100 gm of plasmodium did not involve more time expenditure on the part of the experimenter than the "growth" of the same weight of rabbit muscle.

The sketch in figure 1 represents the type of moist culture chamber used for the propagation of the organism. The mold was harvested daily by scooping it from the surface

[2] For the purpose of brevity it was found necessary to coin a new word. The term "morphoplastic" system or substance, suggested by N. Melechen, was substituted for "a system capable of structural changes under the influence of ATP and related nucleotides."

of the water moat. Fresh oatmeal was added daily and a new culture was started every 3–4 days. The cultures were maintained at a temperature of 23–24°C. Under normal growth conditions one culture would yield 10–25 gm of the organism per day.

The plasmodium was stored at —8°C. in lengths of cellophane tubing (4–5 cm flat width) the ends of which were tied. It was found that such preparations maintained their activity for at least 8 months.

The frozen organism, previously stored at —8°C., was ground in a mortar without added abrasive until a viscous homogeneous liquid was obtained. Fresh material, because of its structural cohesiveness, cannot be ground up with the ease with which previously frozen material can be handled. This liquid was extracted with one to two volumes of 1.2 M KCl containing 0.1 M K_2HPO_4, for 60 minutes at 0–4°C. The pH was usually maintained at 8.1–8.3. The material was then centrifuged at about 2000 $\times$ g for 15 minutes at 4°C. The supernatant was decanted and used in the experiments described below.

Various physical methods could be used for the study of changes in dimension or shape of macromolecules. In this investigation the criterion used for the detection and study of a morphoplastic system was the effect of ATP, and related substances, on the viscosity of the extract. This test was selected because of its simplicity and accuracy. It should be stressed that the viscosity measurements made in this study were used as a means of detecting the occurrence of a morphoplastic effect and not to clarify the molecular basis of the effect.

The viscosity of the extracts was measured in Ostwald viscosimeters with a capacity of 5 ml and the outflow times ranging between 58 and 85 seconds. The viscosimeters were immersed in a bath maintained at 24.85°C. $\pm$ .02. They were calibrated at that temperature by measuring the outflow time of distilled water (t_1). The results were expressed in terms

of the specific viscosity which is $\frac{t_2}{t_1} - 1$, where t_2 is the outflow time of the solution.

Phosphate determinations were made by the Gomori method (Gomori, '42) using a Klett-Summerson photoelectric colorimeter.

Nitrogen determinations were made by a photocolorimetric adaptation of a Nesslerization procedure, using the Bock-Benedict solution (Hawk, Oser, and Summerson, '48).

Adenosine triphosphate (ATP) and muscle adenylic acid (AA5) were obtained from the Ernst Bischoff Co., the former as a di-barium salt, the latter as a free acid.

Muscle inosinic acid (IA5) was purchased from Sigma Chemical Co.

Yeast adenylic acid (AA3), adenine and adenosine were obtained from Schwarz Laboratories.

The barium of the ATP was removed and replaced with sodium by means of an ion exchange reaction on Amberlite resin (Polis, '47). Since passage of the ATP through a column involved some loss and some hydrolysis of ATP, independent determinations after passage through the ion exchange column were made. The method used was based on the assumption that the analytical data supplied with each sample of ATP by the Ernst Bischoff Co. was reliable. Since the analytical data supplied by the company showed the absence of inorganic phosphate and traces of nucleotides other than ATP, and only small quantities (2–4% of total phosphate) of inorganic phosphate were found in the solution after passage through the column, it was felt that measurement of 7 minute phosphate would constitute an adequate method for the determination of ATP.

RESULTS

All the experiments described below were performed with the crude, alkaline KCl extract, the preparation of which has been described above. The salient observation upon which the following series of experiments are based is represented

in figure 2. We shall concern ourselves first with a presentation and explanation of the phenomenon described in figure 2 and then proceed to a treatment of its properties.

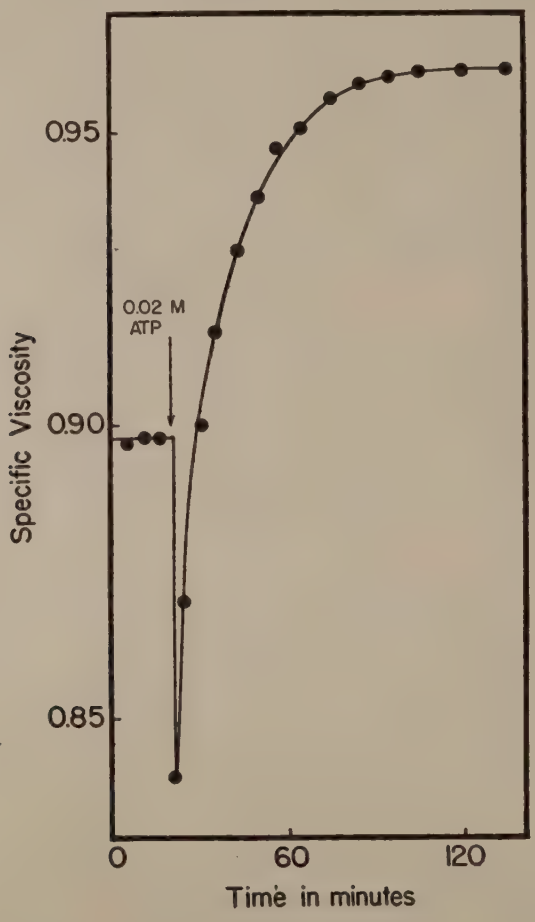

Fig. 2 The effect of a final concentration of 4×10^{-4} M ATP on the specific viscosity of the extract.

I. The morphoplastic effect

(a) The effect. When ATP is added to an extract marked changes in viscosity occur (fig. 2). The curve in figure 2 is composed of two phases, a rapid viscosity decrease fol-

lowed by a slower rise in viscosity which proceeds beyond the initial viscosity of the extract.[3]

(b) A hypothesis to explain the effect. The curve in figure 2 can be explained by assuming that the decrease in viscosity is caused by the ATP and that the subsequent rise is caused by the AA5 produced through the dephosphorylation of ATP. If this hypothesis is true, it should be possible to show that:

1. The extract is able to dephosphorylate ATP and produce AA5.

2. The addition of AA5 itself should cause an increase in the viscosity of the extract.

(c) In support of the hypothesis. A parallel study of two aliquots of an extract is shown in figure 3. The curves represent averages of the data obtained from two samples of extract. ATP was added to the 4 samples and the rate of P increase in two samples was compared with the change in viscosity of the other two samples. The data not only indicate that the extract has apyrase-like activity (removes two phosphates from each ATP molecule) but that the activity is sufficiently high to account for the observed increase in viscosity. From figure 2 it can be seen that the viscosity increase is not instantaneous but requires some 90 minutes to reach a maximum. It might therefore be expected that in figure 3 the viscosity curve should lag behind the P curve and this indeed is what was found to occur.

The data represented in figure 3 constitute proof of ATP hydrolysis but not necessarily AA5 production. It is conceivable, for instance, that a phosphatase might dephosphorylate the AA5 as soon as it appears. That this is not the

[3] The graphical representation in figure 2 is also used in several subsequent figures. In these figures the arrow represents the addition of 0.1 ml of a certain concentration of a substance. The actual concentration of this substance after dilution in the 5 ml of solution present in the viscosimeters is therefore one-fiftieth of the concentration represented above the arrow. Only in the cases where the concentration is variable on the abscissa of a graph (figs. 12–15) are the final concentrations represented.

case is shown in figure 4. The rate at which AA5 is broken down is much lower than that at which it is produced by the breakdown of ATP. The slow rate of AA5 and AA3 breakdown is not due to a generally low phosphatase activity of the extract, since other substrates like hexose diphosphate and β-phosphoglyceric acid seem to be dephosphorylated at

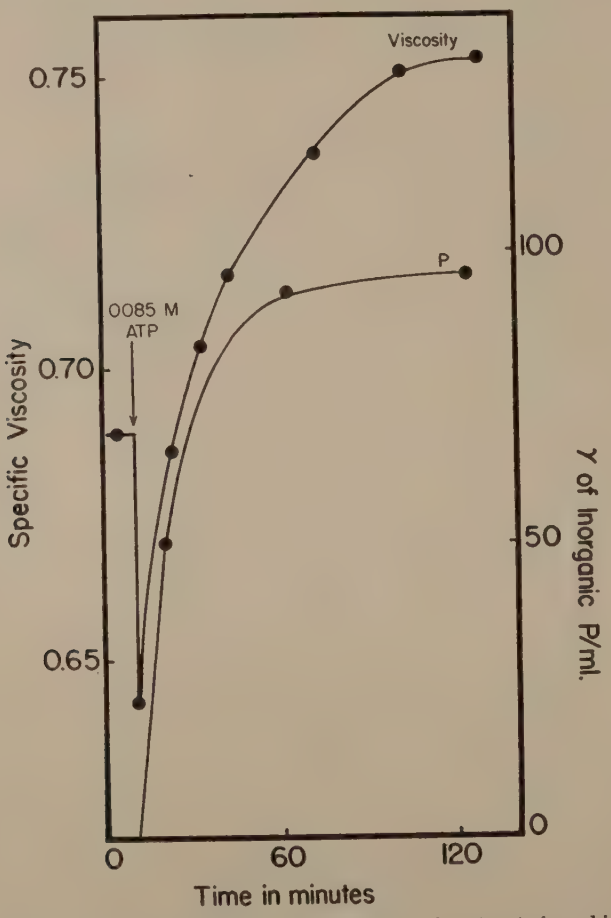

Fig. 3 A comparative study of two aliquots of extract in which ATP is added to final concentrations of .0017 M. The quantitative conversion of ATP to AA5 would account for the appearance of 105 γ of inorganic P/ml. The changes in viscosity of one aliquot are plotted on the same time scale as the increase in inorganic phosphorus in the other aliquot.

a much greater rate (fig. 4).[4] The experiment summarized by figure 4 included a series of controls of boiled extract. The P values represented in the figure are obtained by subtracting the P of the boiled extracts from that of the active

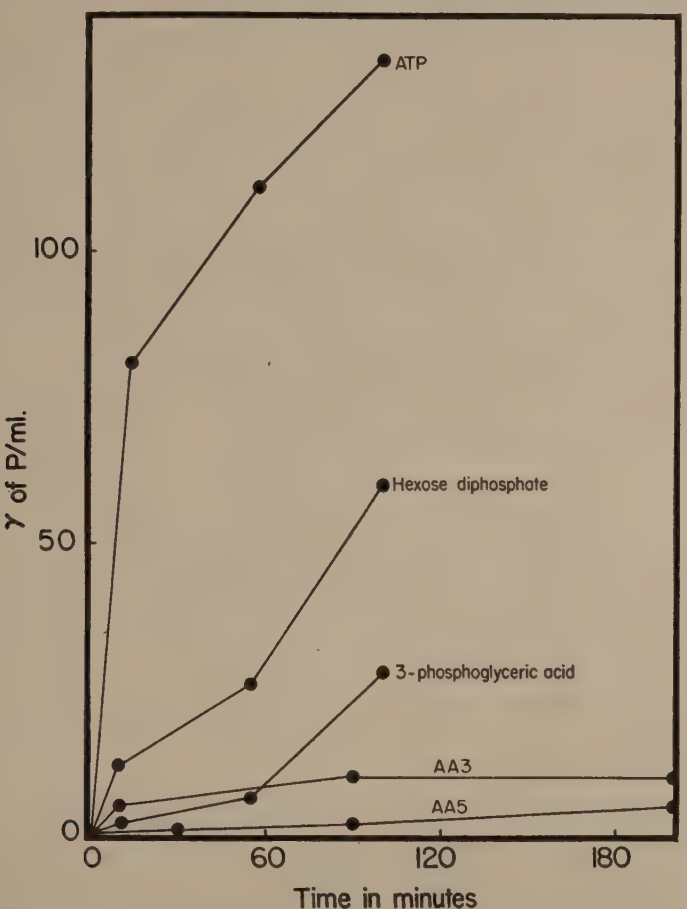

Fig. 4 The dephosphorylation of .0027 M of various substrates by the extract. The quantitative conversion of ATP to AA5 would account for the appearance of 168 γ of inorganic P/ml.

[4] An alternative and more plausible interpretation of the data was suggested by one of the referees who felt that the low rates of AA5 and AA3 hydrolysis indicate a low alkaline phosphatase activity and that the relatively rapid phosphate release from fructose diphosphate and 3-phosphoglyceric acid may occur via glycolysis as suggested by the latent period shown in figure 4.

extracts. This procedure allows not only for non-enzymatic catalysis of the extract but also for the spontaneous decomposition of the compounds used. The latter possibility is of importance in the case of ATP which is somewhat unstable under alkaline conditions. We can conclude from the experiment summarized in figure 4 that when ATP is added to an extract AA5 accumulates in the medium. It is possible that the AA5 produced is modified in some way (for instance, it might be deaminated) but at least it is not dephosphorylated as rapidly as it is generated by the dephosphorylation of ATP.

It remains to be shown that the addition of AA5 itself will cause an increase in viscosity. This is precisely what occurs (fig. 5).

Thus it is felt that the explanation of figure 2 given above is sufficiently justified by the experiments reported to warrant its acceptance in subsequent work.

II. On the significance of the morphoplastic effect

This section is concerned with those properties of the morphoplastic effect which will demonstrate its significance. The criteria used in gauging this significance can be described by the following queries.

(a) Is the effect exerted on a single system or is it the result of a series of diverse phenomena, inseparable by the technique employed?

(b) Is the effect specific or can it be caused by other compounds?

(c) Is it sensitive or does it require abnormally high levels of ATP and AA5, far higher than found *in vivo?*

(d) Is the effect reversible?

These, it is believed, are criteria which might distinguish between a mechanism of fundamental physiological importance and an *in vitro* artifact of dubious significance.

(a) *In support of a single system hypothesis.* The morphoplastic effect described above could conceivably be caused by

at least two systems, one causing a decrease in viscosity on the addition of ATP, the other causing an increase in viscosity on the addition of AA5. An experiment was therefore designed to determine whether the effect of ATP and AA5

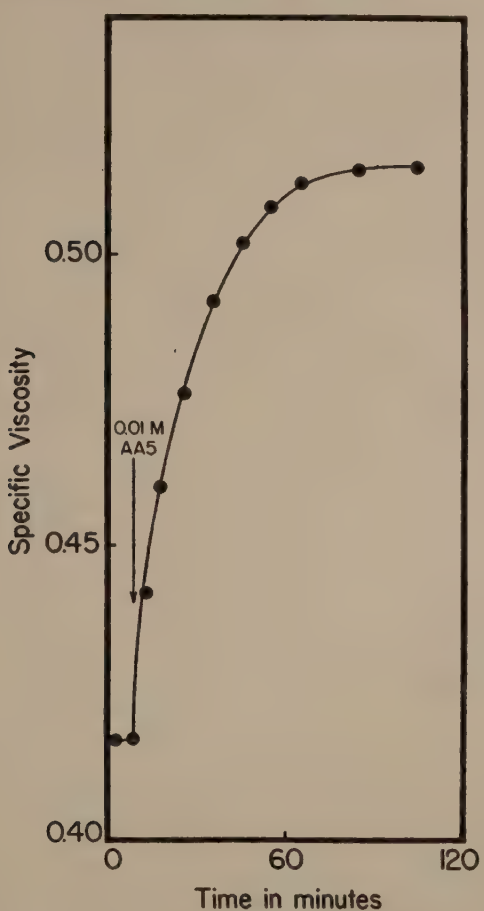

Fig. 5 The effect of a final concentration of 2×10^{-4} M AA5 on the specific viscosity of the extract.

are exerted on the same or on different systems. If we assume that the effect is exerted on two different systems (i.e., that ATP lowers the viscosity of one and AA5 raises the viscosity of the other), it might be expected that the degree of lower-

ing by ATP is independent of the degree of increase by AA5. That this is not the case is proved by the results shown in figure 6. Two equal aliquots of extract were taken and their viscosity was measured for 10 minutes. Then AA5 was added to *a* and ATP was added to *b*. After 90 minutes ATP was added to *a*. The decrease in viscosity caused by ATP was much greater after AA5 was added, indicating that

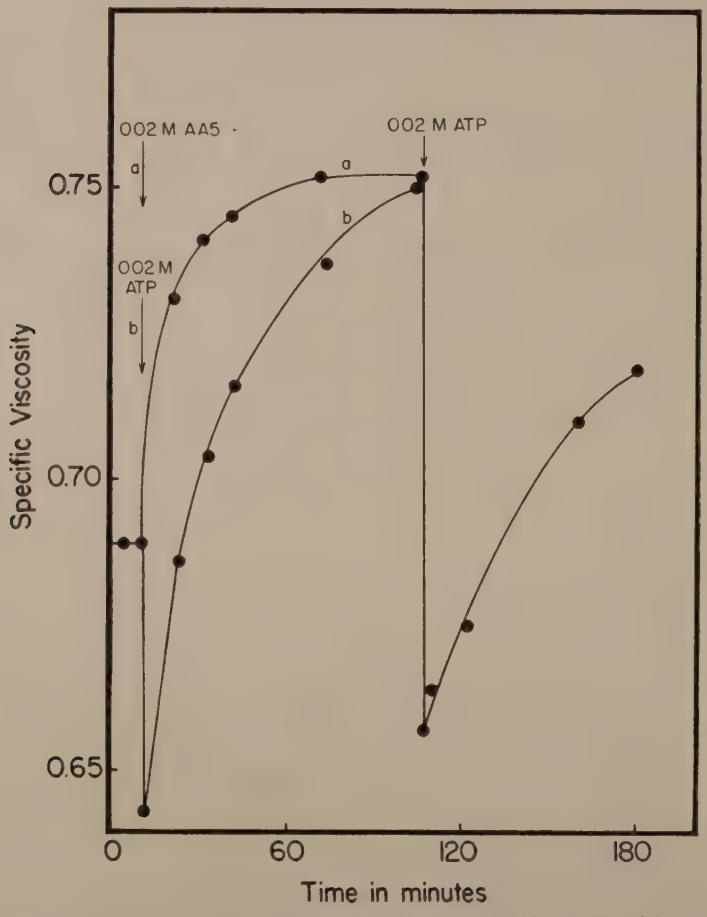

Fig. 6 The effect of final concentrations of 4×10^{-4} M ATP with and without previous treatment of final concentrations of 4×10^{-4} M AA5. To aliquot *a* AA5 is first added and then followed with ATP; to aliquot *b* only ATP was added.

the ATP must have exerted its effect on the system which had previously reacted to the AA5. The reversibility of the morphoplastic effect (fig. 10) is also in keeping with the single system hypothesis.

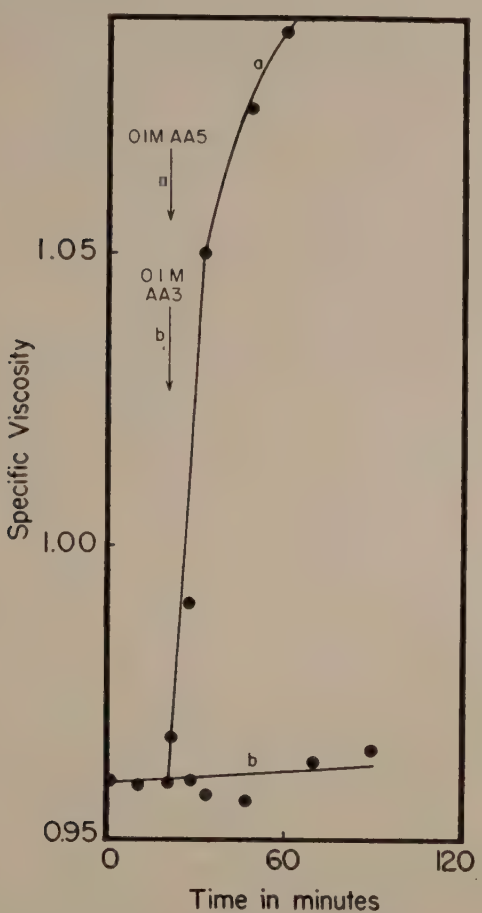

Fig. 7 The lack of activity of yeast adenylic acid (AA3). To aliquot *a* AA5 was added, to aliquot *b* AA3 was added. Final concentration of both reagents was 2×10^{-3} M.

(b) *Specificity.* A striking illustration of the specificity of the effect is shown in figure 7. In this experiment a control was used to which distilled water was added. The change

in viscosity due to dilution was then used as a correction factor in plotting the AA5 and AA3 curves. The slight drop and subsequent rise of the AA3 curve is of no significance since it is within the experimental error of the method. The experiment shows that yeast adenylic acid (AA3), a structural isomer of muscle adenylic acid (AA5), but in no way

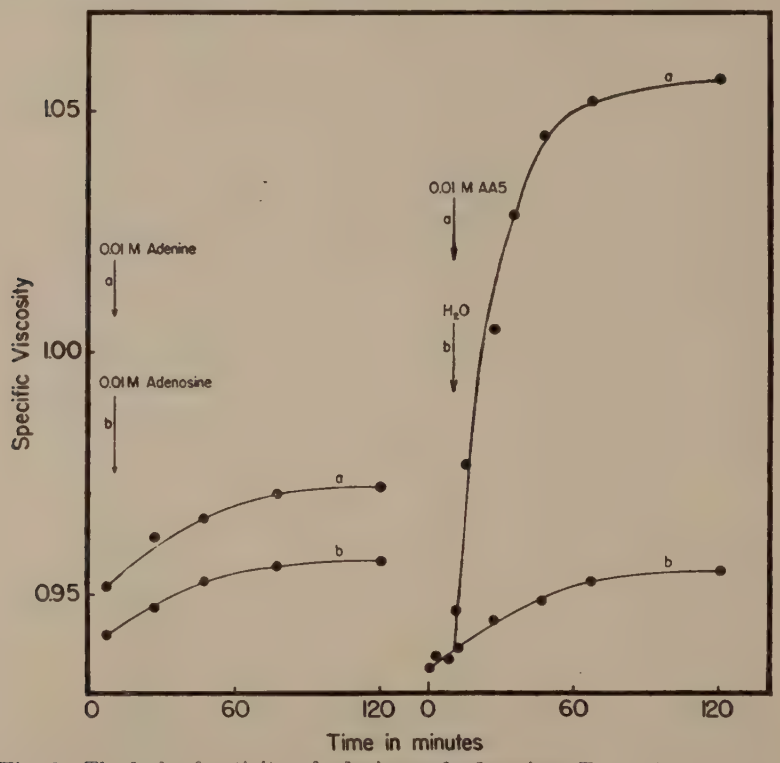

Fig. 8 The lack of activity of adenine and adenosine. To 4 aliquots, adenine, adenosine, water and AA5 were added in final concentrations of 2×10^{-4} M.

physiologically related to the ATP nucleotides, has no effect on the viscosity of the extract. This high specificity involving even the exact position of the phosphate group on the ribose, is taken as the single most convincing indication that the morphoplastic effect is not an *in vitro* artifact but is a special reaction of physiological significance. A study of the effect of adenine and adenosine is summarized in figure 8. It may

be observed that the curves of adenine, adenosine and water are not perfectly horizontal but seem to curve upwards. This phenomenon was encountered occasionally in some of the controls and was found to be due to a pH drift caused by the acid-producing residual metabolism of the extract. Since the adenine and adenosine curves resemble, within the limits of experimental error, the control curve to which only H_2O

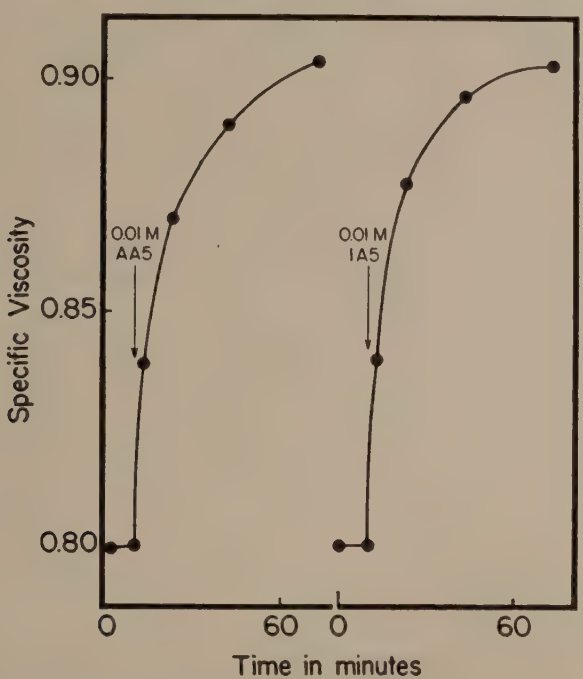

Fig. 9 The similarity in effect of final concentrations of 2×10^{-4} M muscle adenylic acid (AA5) and muscle inosinic acid (IA5).

was added, it can be safely concluded that adenine and adenosine are without morphoplastic activity.

Although the morphoplastic effect shows great specificity with respect to the phosphate grouping on the ribose, it shows no specificity for the presence or absence of the amino group in the 6-position of the purine nucleus. The similarity in action of AA5 and IA5 (muscle inosinic acid) is shown in figure 9. This lack of specificity for the amino group is in

keeping with the results of Bowen and Spicer ('50) who reported that inosine triphosphate (just as ATP) could cause the contraction of muscle actomyosin threads. It is felt that this parallelism is probably more than a mere coincidence.

(c) *Sensitivity.* A measurable increase in viscosity can be obtained with an AA5 concentration of 2×10^{-5} M (fig. 12). Some attempts were made to measure the sensitivity to ATP but for reasons which will become apparent below, they are at best approximate. A large decrease in viscosity can be obtained with a concentration of 2×10^{-4} M ATP (fig. 14). These concentrations are comparable with the level of nucleotides found in tissue and blood (Albaum, '48; LePage, '48) indicating that the morphoplastic effect could be functional *in vivo.*

(d) *Reversibility.* One important requirement for a system functioning *in vivo* is its ability to be reversible or cyclical. A mechanism capable of only one cycle must be considered to be either an artifact or incomplete. An experiment in which the morphoplastic effect was repeated 5 times and showed stability over 5 hours is represented in figure 10. The decreasing mean viscosity might be due to the effect of dilution owing to the repeated addition of 0.1 ml ATP. It should be noted that with each cycle there is a decreasing rate of viscosity increase, whereas the rate of decrease of viscosity remains unchanged.

III. Some general properties of the morphoplastic effect

This section will be concerned with a study of some general properties of the morphoplastic system aimed at a better understanding of its mechanism. It is stressed that the work presented in this section is still in its initial stage and that this section should be considered as a progress report.

(a) *The effect of concentration.* A sample of the data relating the effect of concentration of AA5 to the increase of viscosity is represented in figure 11.

It was found necessary to devise a quantity which will be a measure of the degree of viscosity change. This measure

must have the property of rendering results of different experiments comparable. The "viscosity index" was devised for this purpose.

$$\text{Viscosity index} = \frac{\text{final specific viscosity} - \text{initial specific viscosity}}{\text{initial specific viscosity}} \times 100$$

Its physical meaning is simply the change in viscosity per unit viscosity of extract. If the viscosity index is plotted

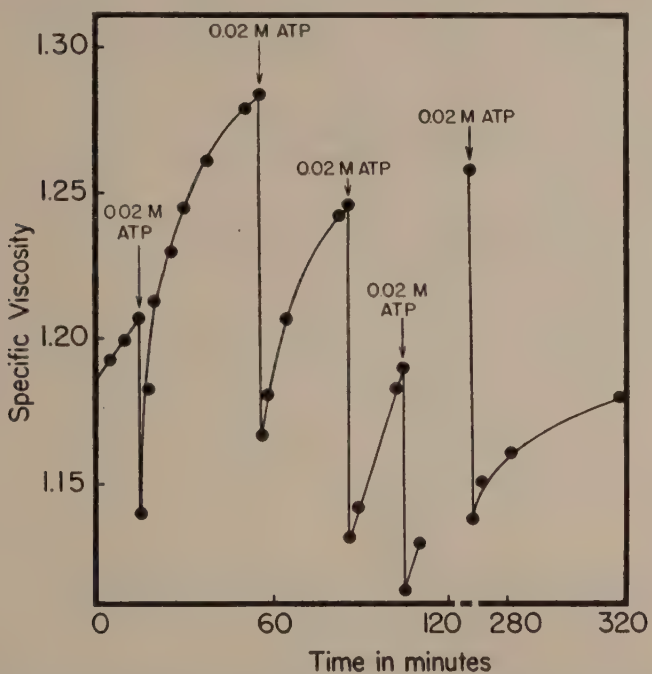

Fig. 10 The reversibility of the morphoplastic effect. Periodic additions of ATP will repeatedly produce the characteristic decrease-increase of viscosity.

against the logarithm of the molar concentration, a straight line is obtained (fig. 12). The slope of this line varies from one extract to another but the concentration at which the line crosses the abscissa seems to vary much less (fig. 12). This concentration may well be a constant for this reaction, a measure of its sensitivity unaffected by the uncontrolled variables which differentiate one extract from another.

The determination of the effect of ATP concentration on the decrease in viscosity is a complicated technical problem.

1. Because of the logarithmic relationship to the effect of AA5, and to its high sensitivity, small amounts of AA5 exert a disproportionate effect. Since all the commercial prep-

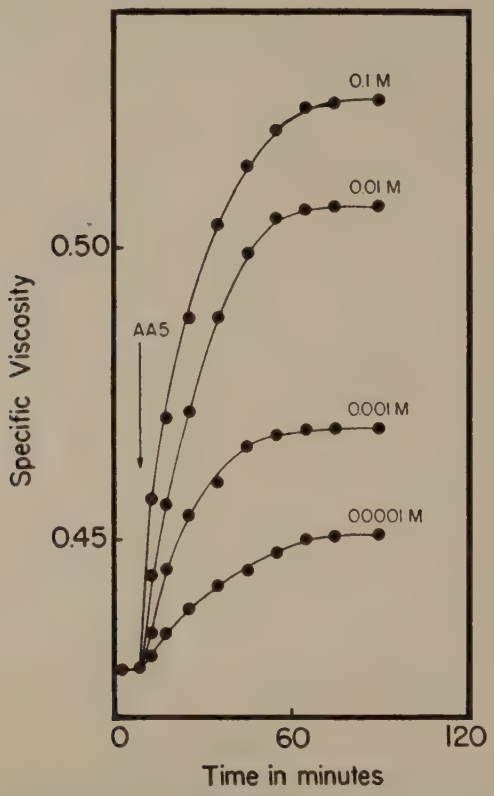

Fig. 11 The effects of various concentrations of AA5. The concentration figures given are those of the AA5 added, the final concentrations being one-fiftieth of the above figure.

arations of ATP contain small amounts of AA5, possible interfering effects of the latter have not been ruled out. This difficulty might be overcome by the utilization of a purer preparation of ATP, possibly synthetic ATP.

2. The viscosity lowering caused by ATP is very quickly followed by an increase in viscosity (fig. 2). Since the vis-

cosimeter outflow time of the solutions used is about two minutes, it seems unlikely that the entire extent of the viscosity drop is ever measured. An instantaneous method of measuring viscosity would obviate this difficulty.

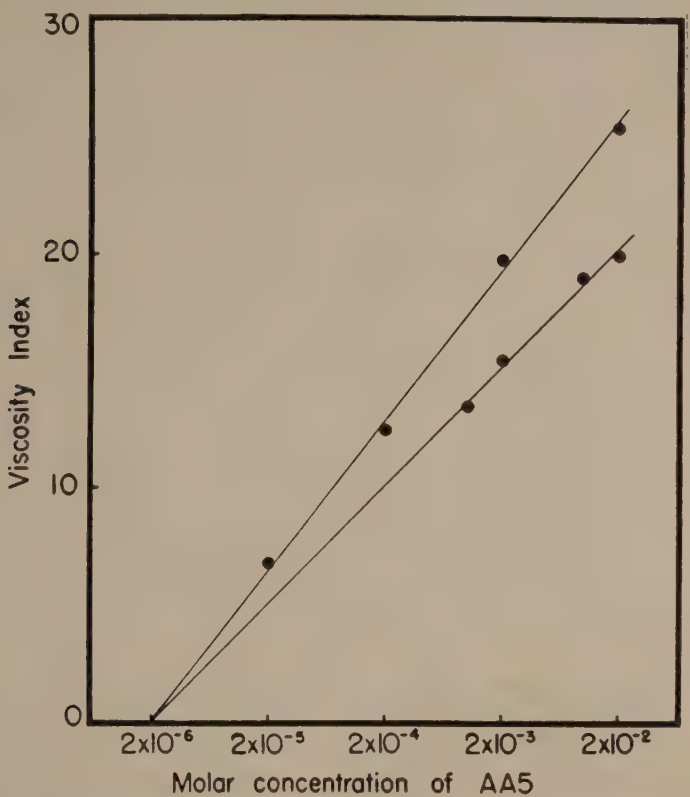

Fig. 12 Relationship between the logarithm of the final AA5 concentration and the viscosity index in two different experiments.

Some preliminary data on the effect of ATP concentration is presented in figure 13. The data are incomplete and should only be considered as a preliminary report. It suggests that there is a threshold effect in the relation between ATP to the morphoplastic effect. The sharpness of the threshold remains to be determined; however, the ATP concentration must lie between $2 \times 10^{-5} - 2 \times 10^{-4}$ M before a viscosity

decrease is obtained. Although the data do not conclusively prove this threshold effect, the latter has such attractive possibilities (see Discussion) that the temptation to present this initial material could not be resisted.

In the interaction of AA5 and ATP two observations based on previous experiments are of interest.

1. The morphoplastic effect is perfectly reversible even though, owing to the addition of ATP and its consequent

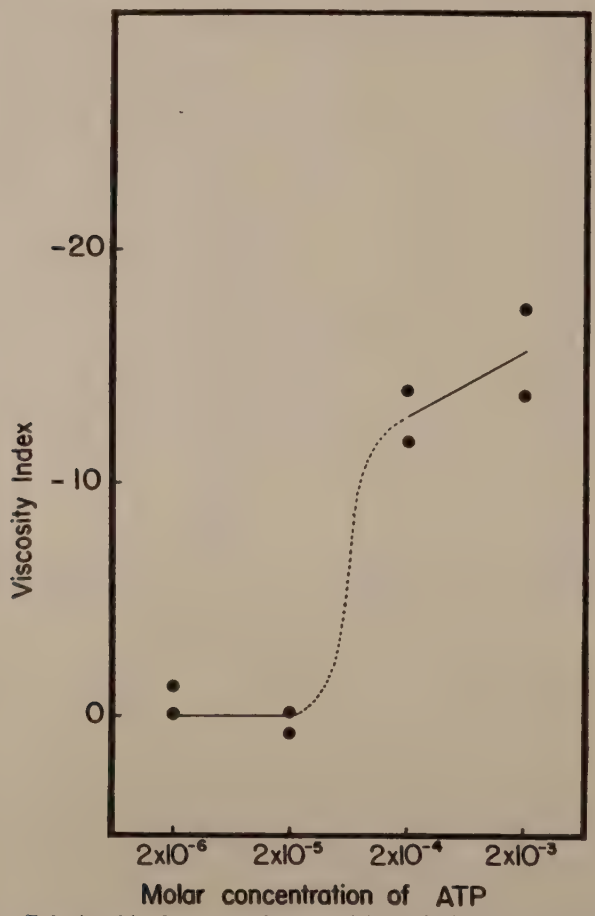

Fig. 13 Relationship between the logarithm of the final ATP concentration and the viscosity index. Since ATP causes a decrease in viscosity, the viscosity index is negative.

dephosphorylation, AA5 accumulates in the medium (fig. 10). It would seem that ATP lowers the viscosity irrespective of the presence of increasing amounts of AA5 in the medium.

2. That the effect of AA5 on viscosity can proceed over a very wide range of concentration ($2 \times 10^{-6} - 2 \times 10^{-2}$ M) is shown in figure 13.

An experiment was designed (fig. 14) to determine whether the effect of the ATP is modified considerably by the AA5

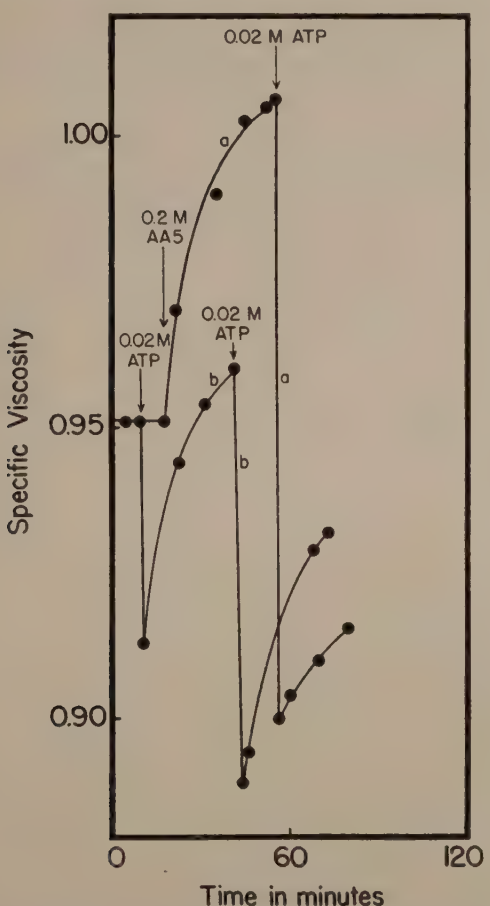

Fig. 14 The effect of ATP on the viscosity of two aliquots containing different amounts of AA5. To aliquot *a* 0.2 M AA5 and subsequently .02 M ATP is added. To aliquot *b* 0.02 M ATP is added in two successive intervals.

level in the medium. Two aliquots of extract were taken. To one of them ATP was added, to the other one (fig. 14) 10 times as much AA5 was added. The results indicate that the final viscosity attained, when lowered with ATP, seems to be approximately independent of AA5 concentration in the medium. On the other hand, the total drop in viscosity is dependent on the previous AA5 concentration because the latter determines the viscosity level from which the lowering occurs.

(b) *An attempt at high-speed centrifugation.* Sedimentation of the morphoplastic system with a high-speed centrifuge should prove a powerful technique for a more detailed study of the morphoplastic system. It would achieve two ends:

1. The purification of the system.
2. The obtaining of data bearing on the size and shape of the morphoplastic complex.

Four aliquots of an extract were taken and to two of them (C and D) AA5 was added. After 25 minutes A and C were centrifuged at 50,000 × g, and B and D were kept as controls. The viscosity of the extract was then measured. AA5 was added to A and B, and ATP to C and D and the effects of these on the viscosity of the extracts was then recorded (table 1).

TABLE 1

High-speed centrifugation at $5 \times 10^4 \times$ gravity of the morphoplastic system

SAMPLE	A	B	C	D
Initial addition of AA5	0	0	4×10^{-4} M	4×10^{-4} M
Treatment	centrif.	control	centrif.	control
Initial specific viscosity	0.660	0.853	0.894	1.079
Final nucleotide concentrations	AA5 4×10^{-4} M	AA5 4×10^{-4} M	ATP 4×10^{-4} M	ATP 4×10^{-4} M
Final specific viscosity	0.721	0.922	0.797	0.950
Viscosity Index	9.2	8.1	— 10.9	— 11.9

The experiment was designed to answer several questions:

1. Is the morphoplastic system, as found in the extract, capable of sedimentation in three hours at 50,000 × g?

2. Is it capable of sedimentation after AA5 has been added?

3. What is the protein content and the specific viscosity of the various fractions, and how do they correlate? (See section c).

The results (table 1) show no significant difference between the viscosity indices of the centrifuged (A, C) and uncentrifuged (B, D) samples. It was thought that the addition of AA5 before centrifugation might increase particle size and hence facilitate sedimentation. But since the viscosity indices of C and D are not significantly different we can conclude that the addition of AA5 does not facilitate the sedimentation of the morphoplastic substance.

(c) *Protein content of fractions.* During the high-speed centrifugation experiment determinations of the protein content of the various fractions were made (table 2). A myxomycete sample was homogenized, two small aliquots of the homogenate were weighed, and total and non-protein nitrogen [5] determinations were made. From this the protein nitrogen was calculated (row 4). From the remaining homogenate 20 gm were weighed out (row 1) and a known volume of extracting liquid (row 2) was added. After extraction and centrifugation at 2,000 × g the supernatant was decanted and its volume was measured (row 3). The total and non-protein nitrogen of an aliquot of supernatant I was measured and the protein content was calculated. From rows 1 and 4 the total protein content of the 20 gm sample was calculated. From rows 3 and 5 the total protein content of the supernatant I was calculated, and from the last two figures the percentage of protein extracted was calculated. Some of the supernatant I was then centrifuged at 50,000 × g for three hours, the total and non-protein content was measured and

[5] Non-protein nitrogen was determined by precipitating the protein with a final concentration of 5% trichloracetic acid and assaying for nitrogen in the supernatant.

the protein content was calculated (row 6). The percentage of protein sedimented at 50,000 × g was then calculated (row 10). Rows 11 and 12 give the specific viscosities of the two supernatants. The data in table 2 were obtained from two different experiments. Experiment I is the same as discussed in section (b). In this experiment duplicate aliquots

TABLE 2

Protein nitrogen and viscosity of various fractions

	EXPERIMENT	I	II
1	Weight of myxomycete sample — gm	20	10
2	Volume of extracting liquid — ml	20	15
3	Volume of supernatant I (2,000 × g)	22	18
4	Milligrams of protein N/gm of organism	4.53, 4.32	4.23
5	Milligrams of protein N/ml of supernatant I (2,000 × g)	1.64, 1.71	1.37
6	Milligrams of protein N/ml of supernatant II (50,000 × g)	1.37, 1.38	1.12
7	Total protein content of sample — mg (1 × 4)	90.6, 86.4	42.3
8	Total protein content of supernatant I (3 × 5)	36.1, 37.6	24.6
9	Per cent protein extraction (8/7 × 100)	40, 43	58
10	Per cent of supernatant-I-protein sedimented at 50,000 × g (100-6/5)	17, 19	18
11	Specific viscosity of supernatant I	0.85	
12	Specific viscosity of supernatant II	0.66	

were taken and found to agree closely. In experiment II only one aliquot was taken and no viscosity determinations were made.

On examination of the data presented by table 2 several facts seem worth noting. The percentage of protein extracted with alkaline KCl solution is surprisingly high (40–58%).

This is a good indication of the degree of impurity of the extract used in these experiments. High-speed centrifugation under the conditions described above does not sediment much protein (17–19%) but accounts for a considerable change in viscosity. This is not surprising since the proteins easily sedimented are the large ones which also contribute disproportionately to the total viscosity of the extract. Because of its marked effect on the viscosity of the extract the failure of the morphoplastic system to sediment is probably not due to a small particle size but rather to considerable particle asymmetry.

(d) *Attempts at purification.* Attempts at purification have thus far failed. If an extract is dialized against the same solution with which it is made (1.2 M KCl — .1 M K_2HPO_4), it loses its morphoplastic activity. The activity cannot be restored by adding boiled alkaline KCl extract or unboiled distilled water extract. These results mean that either a diffusible co-factor is required for morphoplastic activity in the absence of which the system is irreversibly inactivated, or that a diffusible co-factor is required for activity which is heat labile and cannot be extracted with distilled water.

DISCUSSION

It is evident that a system has been extracted from the plasmodium of a myxomycete, the viscosity of which is delicately controlled by at least two substances. The precise change in structure caused by these substances is of course not yet understood, but it is reasonable to assume that they oppose each other in a *common* effect. Thus a very flexible mechanism of control of physical structure is noted, one endowed with a degree of versatility well suited for physiological regulation.

Let us review the properties of the morphoplastic system:

1. The viscosity of this system is increased by AA5.
2. This can occur over a wide range of AA5 concentration.
3. In the same system the viscosity is decreased by ATP.

4. This decrease will occur only after ATP has reached a certain threshold concentration (not yet precisely defined).

5. The dephosphorylation of ATP occurs during the viscosity decrease (an assumption).

6. The AA5 produced is dephosphorylated extremely slowly.

7. ATP is supplied by the metabolism at a sufficiently high rate to permit the building up of the threshold concentration (an assumption).

If we assume 4, 5 and 7, we endow the morphoplastic substance with the properties of a cyclical system, the physical nature of which is controlled by a steady supply of ATP. The unique property of such a system would be the achievement of a *discontinuous* cyclical variation by the addition of a continuous energy supply (fig. 15). If the above assumptions are true, such cyclical behavior might be duplicated *in vitro*.

The wide range over which the AA5 effect is active may be of considerable adaptive significance. Since the ATP and AA5 nucleotides are active in many other reactions, it might be expected that their levels of concentration vary considerably. Therefore a mechanism requiring a critical regulation of both the balance and absolute levels of nucleotides would impose conditions which were too restrictive to the rest of the metabolism. The morphoplastic effect on the other hand seems to be a marvel in that it achieves cyclical variation with dependency on only one non-fluctuating external condition — a rate of ATP supply which exceeds the rate of breakdown sufficiently to permit the accumulation of threshold amounts of ATP. Properties like the threshold response to ATP, the logarithmic response to AA5, the ATP-ase or apyrase activity, the ability to change structurally, are the requirements for a cyclical response and seem to be "built" into the morphoplastic system enabling the latter to respond in so complex a fashion to a very simple external variant.

The reversibility and general stability of the morphoplastic system suggests that it has been extracted without serious damage or loss of components.

What evidence do we have that the morphoplastic effect observed *in vitro* is also operative in the cell? Kriszat ('49) describes the effect of ATP and related compounds on the viscosity of the giant amoeba *Chaos chaos*. His main conclusion is that ATP increases the viscosity of the amoeba. On closer observation of his summary and his experimental results, one finds that ATP first causes a decrease in viscosity lasting 15–45 minutes which is then followed by an increase. Kriszat also tries AA5 (which increases viscosity)

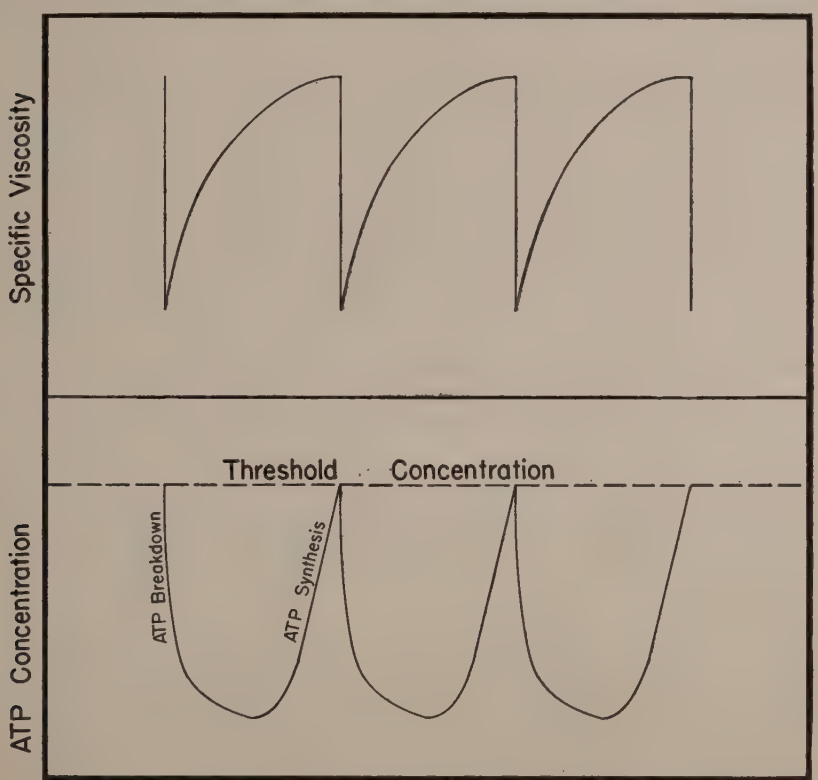

Fig. 15 A scheme illustrating how cyclical variations in viscosity could be achieved by a steady rate of ATP synthesis. It assumes that as ATP is slowly being built up to a threshold concentration the morphoplastic system slowly increases in viscosity due to the presence of AA5 in the medium. At threshold concentration of ATP the viscosity of the morphoplastic system drops suddenly and at the same time ATP is dephosphorylated. While the viscosity rises, the metabolism again raises the ATP to threshold concentration.

but considers its effect "unspecific" and hence not significant. This is an ideal example of both the frequently encountered limitations of purely cell physiological methods, as well as the stultifying effects of the concepts revolving around the relations between "stimulation" and protoplasmic viscosity. It is possible that the homology between Kriszat's results and the *in vitro* results described above is spurious. Nevertheless ATP decreases viscosity of the amoeba before it increases it and since *Chaos chaos* undoubtedly contains enzymes, it seems more reasonable to assume that the earlier effect is the more relevant one.

It is tempting to believe that Kriszat's results involve the same system as the one studied *in vitro* in these experiments. If this is so, a major step in the development of this problem has been achieved, namely the step which links *in vitro* observations to the behavior of the cell as a whole. We would then say that the morphoplastic system extracted here is not only operative *in vivo*, but also considerably effects the physical consistency of the cell as a whole. We are confronted with the possibility that the morphoplastic substance is one of the important structural components of the cytoplasm, the transparent "ground substance" which has hitherto resisted attempts at physico-chemical characterization.

It is apparent that this problem is only in its initial stages of development. To achieve its basic aims it will have to be taken in two directions:

1. Down the scale of integration, to find out about the (a) molecular mechanism of the morphoplastic effect; (b) composition and structure of the morphoplastic unit.

2. Up the scale of integration to find out about the (a) precise way in which the morphoplastic system carried out its work,[6] (b) structural role played by the morphoplastic system in the submicroscopic organization of protoplasm.

[6] Theories relating molecular contractility to mass flow of protoplasm have recently been proposed (Frey-Wyssling, '49; Loewy, '49).

SUMMARY

1. A morphoplastic system has been extracted from the plasmodium of the myxomycete *Physarum polycephalum*.

2. The viscosity of this system is rapidly lowered by adenosine triphosphate and more slowly raised by muscle adenylic acid. The lowering caused by the adenosine triphosphate is followed by a slow rise is viscosity which is interpreted as due to the formation of muscle adenylic acid by the dephosphorylation of the adenosine triphosphate.

3. The lowering of the viscosity seems to occur only above a threshold concentration of $2 \times 10^{-5} - 2 \times 10^{-4}$ M.

4. The viscosity increase occurs over a very wide range of muscle adenylic acid concentrations ($2 \times 10^{-6} - 2 \times 10^{-2}$ M).

5. The changes in viscosity are reversible.

6. The viscosity response is highly specific, being restricted to ATP and some of the nucleotides related to it. Thus yeast adenylic acid, a close isomer of muscle adenylic acid but metabolically unrelated to it, is entirely without activity.

7. It is thought that with the above mentioned properties a steady supply of adenosine triphosphate could cause the morphoplastic system to undergo cyclical alterations in its structure.

8. It is believed that the viscosity changes measured in the extract may be shown to represent a system playing a role in the mechanical work functions of the organism.

I wish to express my appreciation to Professor William Seifriz and Professor David R. Goddard for their valuable advice and friendly encouragement.

LITERATURE CITED

ALBAUM, H. Personal communication.

BOWEN, W. J., AND S. S. SPICER 1950 Superprecipitation of actomyosin by inosine triphosphate. Federation Am. Soc. Exp. Biol., *9:* 406.

FREY-WYSSLING 1949 Physiochemical behaviour of cytoplasm. Research (London), *2:* 300–307.

GOMORI, G. 1942 A modification of the colorimetric phosphorus determination for use with the photoelectric colorimeter. J. Lab. Clinical Med., *27:* 955–960.

HAWK, P. B., B. L. OSER AND W. H. SUMMERSON 1948 Practical Physiological Chemistry, p. 1230. Blakiston Co.

KRISZAT, G. 1949 Die Wirkung von Adenosinetriphosphat auf Amöben (Chaos chaos). Arkiv für Zoologi, *1:* 81–86.

LEPAGE, G. A. 1948 Phosphorylated intermediates in tumor glycolysis. Cancer Research, *8:* 193–196.

LOEWY, A. G. 1949 A theory of protoplasmic streaming. Proc. Am. Phil. Soc., *93:* 326–329.

POLIS, D. 1947 Studies on adenosine triphosphatase in muscle. I. Concentration of the enzyme on myosin. J. Biol. Chem., *169:* 1–13.

SZENT-GYÖRGYI, A. 1951 Chemistry of muscular contraction. Academic Press, 2nd ed.

WEBER, H. H., AND H. PORTZEHL 1952 Muscle contraction and fibrous muscle proteins. Advances in Protein Chemistry, *7:* 161–252.

COMMENTS AND COMMUNICATIONS

Comments relating to articles which have recently appeared in the Journal of Cellular and Comparative Physiology and brief descriptions of important observations will be published promptly in this Section. Preliminary announcements of material which will be presented later in more extensive form are not desired. Communications should not in general exceed 700 words.

CYTOCHROME C OXIDASE ACTIVITY OF VARIOUS TISSUES OF THE AMERICAN COCKROACH, PERIPLANETA AMERICANA (L.)

BERTRAM SACKTOR AND DIETRICH BODENSTEIN
Medical Laboratories, Army Chemical Center, Maryland

ONE FIGURE

In the intermediate metabolism of insects, as in other animals, the cytochrome *c* oxidase system is of vital importance. Although this enzyme system has recently been studied in several aspects, most investigators have employed the entire insect. In order to gain a better understanding of the physiological mechanisms in which this system is involved it is desirable to know the distribution of this enzyme (or enzymes) in the different tissues. To this end a comparative study of the activity of the cytochrome *c* oxidase in the various tissues of the American cockroach, *Periplaneta americana*, has been made.

Adult male and female roaches were dissected in physiological salt solution and the desired tissues were removed. The gut parts were slit open and freed of their food contents by thorough washing. The hind gut preparations contained the rectal glands. The hearts had portions of the alary muscles attached. The remaining organs were removed relatively free of foreign tissues, but the abundant intertwining tracheae could not be separated completely from the fat bodies and accessory glands. However, the amount of tracheae as compared to the amount of tissue tested was negligible. The reproductive parts of the female were not tested because their condition of development varied.

TABLE 1

Cytochrome C oxidase activity of American cockroach tissue

TISSUE	STANDARD ACTIVITY [1] Female	STANDARD ACTIVITY [1] Male	GAMMA N/ MG WET WT. Male	ACTIVITY/ MG N Male
Nerve cord	1.19 ± .09	0.72 ± .05	11.2	2.11
Brain	1.56 ± .13	0.89 ± .07	9.0	3.38
Muscle	1.60 ± .15	2.93 ± .40	17.2	6.80
Heart	2.57 ± .29 ø	2.14 ± .37	25.0	2.55
Fat body	0.59 ± .10 ‡	0.82 ± .08	27.8	0.87
Testes		0.54 ± .05	12.2	1.39
Acc. Gld.		0.47 ± .06	42.1	0.36
Fore gut	1.37 ± .09	0.94 ± .10	8.6	2.02
Mid gut	1.49 ± .18	1.41 ± .17	19.1	2.38
Hind gut	1.20 ± .11 ‡	1.29 ± .17	11.0	4.08
Malp. tub.	0.96 ± .08 ‡	0.96 ± .14	20.4	1.44

[1] $\frac{d \log (Cy\ Fe\ ++)}{d\ t} \times \frac{\text{final tissue dilution}}{100} \pm$ Standard Error.

Each datum is the average of 10 determinations except ‡ 8 determinations, ø 7 determinations.

After preparation each tissue was immediately blotted on filter paper, weighed on a Roller-Smith Precision Balance to the nearest 0.01 mg and then homogenized in iced 0.03 M phosphate buffer, pH 7.4. The cytochrome *c* oxidase activity was measured spectrophotometrically (Sacktor, '50). Separate determinations were made on the tissues from each of 10 roaches. For some tissues fewer roaches were used, as in-

dicated in table 1. Nitrogen analyses were made by the micro-Kjeldahl method of Folin and Farmer ('12). Standard enzyme activities were calculated according to Cooperstein and Lazarow ('51). The results are shown in table 1.

Table 1 shows clearly that the various tissues have different cytochrome *c* oxidase activity, which may be related to their metabolic performance. Furthermore, the oxidase activity of a given tissue may differ, in some cases, with the sex of the animal. Thus, the enzyme activity of the nerve cord, brain and fore gut was greater in the female than in the male. These differences are significant beyond the .01 level.

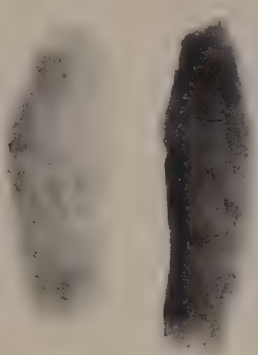

Fig. 1 Identical coxal muscles of adult American cockroach. Left: muscle of female, right: muscle of male. Note deeper coloration in male muscle as compared to female.

On the other hand, the oxidase activity of the muscles in the male was significantly ($P = .01$) higher than in the female. Moreover, it was observed that the muscles of the male were red in color whereas the females' muscles were much lighter, almost white (fig. 1). Slight gradations in the amount of pink were found in the muscles of different females. These variations were reflected in the cytochrome *c* oxidase activity, since muscles with more coloration had a higher, those with less, a lower, activity. Similar though less pronounced differences in color were also found between the

sexes in the nymphs. The contrast between the cytochrome *c* oxidase activity in male and female muscles agrees well with the findings of Barron and Tahmisian ('48). They reported that male cockroach muscles had a higher O_2 consumption and greater content of glycogen, diphosphopyridine nucleotide, diphosphothiamine, cytochrome *c* and iron than did the female.

No significant differences between the sexes existed in the oxidase activity of the mid gut, hind gut, malpighian tubules, heart or fat body. It will be noted that the oxidase activity of the fat body is rather low as calculated either on wet weight or nitrogen basis. This could be due to the large amounts of fat and urates found in this tissue.

A comparison of the data in table 1 with recent observations by Day ('51) is of interest. He found that the tracheation of American cockroach tissues, in order of decreasing abundance, is roughly: muscle, rectum, mid gut, hind gut, nerve, heart, malpighian tubules and fat body. The correspondence between these observations and the cytochrome *c* oxidase activities (per milligram N) is striking. The only tissue with an unexpectedly high metabolic potential, as indicated by its tracheation and oxidase activity, is the rectum (hind gut). Yet it is known (Wigglesworth, '47) that the rectum is concerned with the resorption of materials from the alimentary canal. The facts suggest that the resorption mechanism may require a large expenditure of energy, which is obtained from oxidative processes.

LITERATURE CITED

Barron, E. S. G., and T. N. Tahmisian 1948 The metabolism of cockroach muscle *(Periplaneta americana)*. J. Cell. and Comp. Physiol, *32:* 57–76.

Cooperstein, S. J., and A. Lazarow 1951 A microspectrophotometric method for the determination of cytochrome oxidase J. Biol. Chem., *189:* 665–670.

Day, M. F. 1951 Studies on the digestion of wool by insects. III. A comparison between the tracheation of the midgut of *Tineola* larvae and that of other insect tissues. Austral. J. Scientific Res., *B 4:* 64–74.

FOLIN, O., AND C. J. FARMER 1912 A new method for the determination of total nitrogen in urine. J. Biol. Chem., *11:* 493–501.

SACKTOR, B. 1950 A comparison of the cytochrome oxidase activity of two strains of house flies. J. Econ. Ent., *43:* 832–838.

WIGGLESWORTH, V. B. 1947 The principles of insect physiology. 3rd Ed. Methuen and Co., Ltd., London.

POTASSIUM LOSS IN RABBIT LEUKOCYTES IN RESPONSE TO MECHANICAL AGITATION

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ONE FIGURE

In the course of investigations on the K-Na interchange in rabbit leukocytes, it was observed that the rates of K loss differed markedly depending upon the technique used to maintain the cells in homogeneous suspension.

In view of the fact that these observations reflect the sensitivity of leukocytes to mechanical forces similar to those attained in routine manometric studies, it was felt that a brief resume of the results would be of value.

PROCEDURE

Cells were obtained by the method of de Haan ('20), with the additional modification that a saline-PO_4 solution (one part of 0.11 M phosphate buffer to 9 parts of 0.95% sodium chloride solution at pH 7.2) was used as the lavage solution. The pH of the exudate obtained was 7.5–7.6. Heparin was used as an anticoagulant. Aseptic conditions were observed.

Two methods for maintaining homogeneous suspensions were used. In the first set-up, a rack equipped to hold three 500 ml Erlenmeyer flasks was attached by a series of clamps to a manometer-type shaking apparatus so as to allow the flasks to shake back and forth in a water-bath maintained

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at 37°C. The vessels moved through an arc of 1.4 cm at a rate of 120 oscillations per minute. The movements were of such a nature as to produce a sloshing motion of the liquid, which resulted in a foaming at the surface.

The second method utilized a rolling type of tonometric apparatus in which liter size pyrex bottles were placed in a sloping position on a pair of rolling drums so as to rotate in a direction counter to the drum movement. The motion of the liquid in this case was a uniform flow along the lower sides and bottom of the bottle, with the complete absence of sloshing and foaming.

Duplicate aliquot samples of 40 cm^3 were taken from the shaken and rolled suspensions, at various time intervals. The cells were concentrated in large centrifuge tubes. They were then transferred to 1 cm^3 centrifuge tubes for packing in the air turbine (Parpart and Green, '51). Wet weights were obtained by difference. These packed cells were frozen-dried in the air turbine tubes and the dry weight and per cent water determined. Values for intracellular water remained constant over a 7 hour period. Over a 24 hour period, there was a 5% increase in cell water. The average per cent water was 79.0 ± 1.4, which is in agreement with values reported by Wilson and Manery ('49).

On the average, the air turbine tubes contained ca. 0.1 gm wet cells. These packed cells were cytolized in a known volume of distilled water, shaken intermittently for 30 minutes, and the cell debris centrifuged down. Potassium analyses were made on the supernatant, using a Perkin-Elmer flame photometer.

RESULTS

Figure 1 shows the rate of K loss over a 24 hour period when the cells were kept suspended by the two methods. The differences are quite apparent. With shaking, the K content of the cells approached diffusion equilibrium by 12 hours. In contrast, cells suspended by the roller technique lost only 6% during the same time period.

The sigmoid shape of the curve of K loss with shaking may have at least two qualitative explanations:

1. It expresses the rate at which cells are being damaged in the air bubble film, caused by foaming. This would cause a rapid loss of K along a diffusion gradient of 40:1.

2. It is the summation of the gradual change of rate of K loss over the entire population of cells, with respect to time,

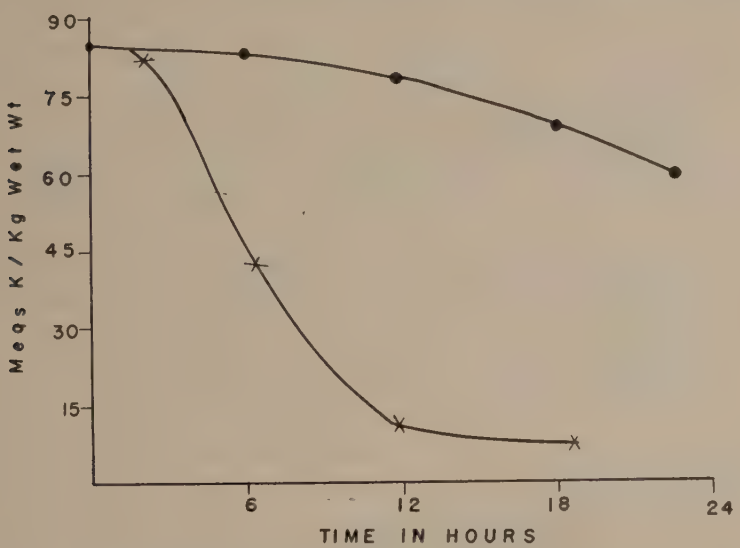

Fig. 1 Potassium loss with mechanical agitation, 37°C. with external K = 2 meq/1; ● = tonometer; x = shaker.

in response to mechanical stress. Which of these two actions predominates, or whether they occur simultaneously, cannot be ascertained at this point.

It is interesting to note that Hartman ('52) in a study of the effect of leukocyte concentration on O_2 consumption, using a Warburg, shaking, manometric technique, found that O_2 consumption tends to fall off after three hours. From figure 1 it is evident that shaken leukocytes begin to lose large amounts of K after three hours. If the degree of mechanical agitation in our experiments is in any way comparable with those

reported by Hartman, it may be inferred that the decrease in O_2 is correlated with mechanical injury.

It is necessary, therefore, in any study of physiological activity of leukocytes, to bear in mind the extreme sensitivity of these cells to technical manipulation. In this connection, a roller type of technique for homogeneous suspension is much to be preferred for such studies as ion exchange or respiration, over long periods of time.

LITERATURE CITED

DE HAAN, J. 1920 Doctorate Thesis, Gröningen. Quoted by A. Bakker, 1927. Quelques différences entre des cellules d'exsudat polynucléaires et mononucléaires. Arch. néerland de physiol., *11:* 234.

HARTMAN, J. D. 1952 Effect of cell concentration on O_2 consumption of leukocytes under varying conditions. Proc. Soc. Exp. Biology and Medicine, *79:* 3.

PARPART, A. K., AND J. W. GREEN 1951 Potassium and sodium exchanges in rabbit red cells treated with n-butyl alcohol. J. Cell. and Comp. Physiol., *38:* 347.

WILSON, D. L., AND J. F. MANERY 1949 The permeability of rabbit leukocytes to sodium, potassium, and chloride. J. Cell. and Comp. Physiol., *34:* 493.

CHOLINESTERASE CONTENT OF VARIOUS SKELETAL MUSCLES AFTER DENERVATION

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The well-known fact that a denervated organ becomes supersensitive to certain substances, especially those that normally transmit excitation at its neuroeffector junctions, has never been satisfactorily explained. The explanation might be sought in a change of enzyme content: thus, the increase in sensitivity to acetylcholine (ACh) that occurs in structures deprived of their cholinergic innervation might be ascribed to the loss of

cholinesterase (ChE) that takes place at the same time. Cannon and Rosenblueth ('49) have pointed out, however, that when a number of tissues are compared the extent of ChE loss is not well correlated with the degree of supersensitivity. For instance, skeletal muscles are sensitized much more than autonomic ganglia, yet lose less of their ChE. Furthermore, skeletal muscles do not all show the same changes after denervation. Brooks and Myers ('52) who have reviewed the literature on this subject, found that the ChE content of the denervated *M. serratus anterior* of the guinea-pig was within the normal range, although the muscles were always supersensitive to ACh. Other workers, examining different muscles in the guinea-pig and other species, have observed changes ranging from an increase to a loss of up to two-thirds of the ChE.

The present communication confirms the results of Brooks and Myers on the guinea-pig serratus and reports in addition the effect of denervation on the gastrocnemius of guinea-pig and rat. All assays were carried out manometrically on muscle tissue that had been ground with sand. Details of the method are given by Burgen and Chipman ('51). The weights and enzyme concentrations for the same groups of muscles are listed in tables 1 and 2 respectively. The data for rat gastrocnemius are presented in full because the scatter of values would not be apparent from the means alone. The values for the 4 post-operative intervals do not differ significantly and the group means for all animals are therefore given in table 2. It can easily be seen that when gastrocnemius muscles of either species are examined 12–27 days after denervation, the true ChE content is found to have decreased roughly in proportion to the loss in fresh weight. In fact, when the ratio "Denervated/Normal" is calculated for each rat, for muscle weight on the one hand and for muscle enzyme content on the other, the means for the two sets of ratios are found not to differ significantly ($t = 2.4$, $P = 0.05$).

Thus the behavior of ChE after denervation in gastrocnemius and in serratus anterior is different, and cannot ac-

TABLE 1

Weights and ChE contents of denervated guinea-pig's muscles

DAYS POSTOP.	NO. OF ANIMALS	MUSCLE	NORMAL MUSCLE Weight Mean ± S.E.	DENERVATED MUSCLE Weight Mean ± S.E.	DENERVATED / NORMAL Mean ± S.E.	NORMAL MUSCLE ChE content Mean ± S.E.	DENERVATED MUSCLE ChE content Mean ± S.E.	DENERVATED / NORMAL Mean ± S.E.
			mg	*mg*		*µl CO_2/10 min./muscle*		
12	6	Gastrocnemius	494 ± 48	310 ± 37	0.64 ± 0.05	65 ± 6	38 ± 5	0.59 ± 0.02
25	5	Gastrocnemius	625 ± 72	305 ± 55	0.50 ± 0.06	68 ± 9	36 ± 4	0.54 ± 0.05
17	6	Serratus anterior [2]	112 ± 20	112 ± 32	0.64 ± 0.14	23 ± 4	23 ± 8	0.98 ± 0.26

[1] Evaluated as means of individual ratios.
[2] Second and third digitations only.
S.E. Standard error of the mean.

TABLE 2

Weights and ChE contents of denervated rat's gastrocnemius muscles

DAYS POSTOP.	NO. OF ANIMALS	NORMAL MUSCLE Weight	Mean	DENERVATED MUSCLE Weight	Mean	DENERVATED / NORMAL Ratio	Mean	NORMAL MUSCLE ChE content	Mean	DENERVATED MUSCLE ChE content	Mean	DENERVATED / NORMAL Ratio	Mean
		mg	*mg*	*mg*	*mg*			*µl CO_2/10 min./muscle*					
17	2	874		413		0.473		57		17		0.30	
		937	905	311	362	0.332	0.403	32	45	22	20	0.69	0.50
20	2	973		435		0.447		67		24		0.36	
		1190	1082	383	409	0.322	0.385	71	69	23	24	0.32	0.34
23	2	715		268		0.375		52		16		0.31	
		940	827	332	300	0.353	0.364	64	58	29	23	0.45	0.38
27	2	899		312		0.347		68		23		0.34	
		967	934	255	284	0.264	0.306	59	64	16	20	0.27	0.31
Totals:	Means	936		339		0.365		59		21		0.38	
	± S.E.	± 49		± 130		± 0.021		± 13		± 5		± 0.14	

[1] Evaluated as means of individual ratios.
S.E. Standard error of the mean.

count for the supersensitivity to ACh that occurs in both muscles. In any case, the idea that the supersensitivity is due to ChE loss is contradicted by the experiments of Brown, Burns and Feldberg ('48) who showed that the cat's tibialis anterior remains normally sensitive to intra-arterially injected ACh after previous injection of di*iso*propyl fluorophosphate (DFP) in dosage sufficient to inactivate practically all the ChE in the muscle. All these results on skeletal muscle stand in contrast to those obtained by Burn and Robinson ('52) on the denervated smooth muscle of the cat's nictitating membrane: they found the amine oxidase concentration of individual muscles to be inversely correlated with their sensitivity to noradrenaline.

LITERATURE CITED

Brooks, V. B., and D. K. Myers 1952 Cholinesterase content of normal and denervated skeletal muscle in the guinea-pig. J. Physiol., *116:* 158.

Brown, G. L., Burns B. Deslisle and W. Feldberg 1948 The effect of di-*iso*propyl fluorophosphonate on neuromuscular transmission in cats. J. Physiol., *107:* 346.

Burgen, A. S. V., and L. M. Chipman 1951 Cholinesterase and succinic dehydrogenase in the central nervous system of the dog. J. Physiol., *114:* 296.

Burn, J. H., and Judith Robinson 1952 Effect of denervation on amine oxidase in the nictitating membrane. J. Physiol., *116:* 21 P.

Cannon, W. B., and A. Rosenblueth 1949 The Supersensitivity of Denervated Structures, p. 195. McMillan, New York.

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